APPLICATION OF MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION, ENZYME-LINKED IMMUNOSORBENT ASSAY AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY TO FORENSIC TOXICOLOGY

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To My Late Parents,

Harun Maan

and

Bedah Md. Shah

Summary

The rapid growth of ketamine and amphetamine misuse worldwide has led to the development of methods for the detection and analysis of ketamine and amphetamines in biological specimens. Most methods previously developed in forensic toxicology for the detection of ketamine and amphetamines used GC-MS. The present work developed alternative methods based on LC-MS/MS. Ketamine was chosen as the drug of interest because there are no data currently available on the extent of ketamine abuse in Malaysia even though a large amount of ketamine has been seized by the Malaysian Royal Police, while amphetamines are the most widely abused synthetic drugs in South East Asia including Malaysia.

The study addressed some of the challenges facing the forensic toxicologist, such as the need to use new technology (LC-MS/MS) and improve sensitivity and selectivity in forensic toxicology analysis through efficient sample preparation techniques. The general requirements of method validation, including as the parameters linearity, limit of detection (LOD) and Lower Limit of Quantification (LLOQ), recovery, precision and matrix effects were observed. Three main techniques were used in the study: enzyme-linked immunosorbent assay (ELISA), liquid chromatography tandem mass spectrometry (LC-MS/MS) and molecularly imprinted polymer solid phase extraction (MISPE). MISPE is a new extraction technique in forensic toxicology applied to biological samples.

Initially work was carried out on the optimization, development and validation of the Neogen® ELISA for screening ketamine and norketamine in urine. The Neogen® ketamine ELISA kit was found to be adequately sensitive and precise for ketamine screening at a cut-off concentration of 25 ng/mL. The ELISA test was shown to be highly specific to ketamine and demonstrated minimal (2.1%) cross-reactivity to its main metabolite norketamine compared to ketamine.

Subsequently, an LC–MS/MS confirmation method for ketamine and norketamine in urine samples was developed and validated with application of the method to urine samples from chronic ketamine users in Malaysia. The method demonstrated good linearity, LOD, LOQ, accuracy and precision and had acceptable matrix effects.

The efficiency of ELISA as a screening method at cut-off of 25 ng/ml and LC-MS/MS as a confirmation method at 2 ng/ml was evaluated. These methods complemented each other and both ELISA and LC-MS methods were 100% sensitive and specific with no false positive results for ketamine and norketamine in urine samples. The results demonstrated that a combination of these two methods can be reliably used for routine screening and confirmation of ketamine and norketamine in urine specimens. Preliminary data from this study provided information on the concentrations of ketamine and norketamine typically found in urine samples collected from individuals frequenting pubs in Malaysia.

The main work in this thesis involved molecularly imprinted polymer materials which were used as sorbents in solid phase extraction (MISPE). Ketamine was used as a model substance for novel in-house synthesised MIPs as no anti-ketamine MIP have previously been reported and because the ketamine structure is suitable for the synthesis of molecularly imprinted polymers. The study was intended to improve the selectivity and sensitivity of the extraction method (MISPE) prior to LC-MS/MS analysis. Evaluation of polymer imprinting was carried out using HPLC-UV.

MIP extraction and LC-MS/MS analysis were applied to the determination of ketamine and norketamine in hair samples and compared with a conventional SPE-based method. MISPE extraction was selective and sensitive with fewer matrix effects than the conventional SPE method and could also be applied to norketamine, the principal metabolite of ketamine, due to the group-selective binding nature of the MIP, but not to structurally dissimilar analytes such as PCP and tiletamine. MISPE was superior to conventional SPE for trace detection of ketamine and norketamine in hair, in terms of improved sensitivity, lower limits of detection and reduced matrix effects.

In addition, the commercial product Amphetamine SupelMIP^{TM} was evaluated for identification of amphetamines in post mortem blood coupled with LC-MS/MS analysis. This work assessed whether the MIP, sold by the manufacturer for the extraction of amphetamines in urine, could also be used for whole blood. The results demonstrated that the MIP can be used successfully for the determination of amphetamines in post mortem blood. The recoveries of five

amphetamines were lower than with a comparable GC-MS method but the LODs and LLOQs of the LC-MS/MS method were better and suitable for detection of low levels amphetamines in post mortem blood. Further optimisation is needed to develop an improved protein precipitation method prior to MISPE.

Liquid Chromatography Electro-Spray Ionization Mass Spectrometry (LC–ESI-MS) was used with the MISPE and SPE methods for detection and quantification of ketamine, norketamine and amphetamines in urine, whole blood and hair samples. LC-ESI-MS was found to be easy to use and could detect lower concentrations of drugs and gave reproducible results for all the methods developed in this thesis.

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Abbreviations

AIBN	2,2'-Azobisisobutyronitrile
APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric Pressure Ionisation
CEDIA	Cloned Donor Enzyme Immunoassay
CNS	Central Nervous System
DFSA	Drug-facilitated Sexual Assault
DHNK	Dehydronorketamine
DOA	Drugs of Abuse
DUID	Driving Under the Influence of Drugs
EGDMA	Ethylene Glycol Dimethacrylate
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
FAB	Fast Atom Bombardment (FAB),
FPIA	Fluorescence Polarization Immunoassay
GC	Gas Chromatography
GC/FID	Gas Chromatography with Flame Ionization Detection
GC/NPD	Gas Chromatography with Nitrogen Phosphorus Detection
GC-MS	Gas Chromatography-Mass Spectrometry
hCG	Human Chorionic Gonadotrophin
HCl	Hydrochloric Acid
HPLC/UV	High Performance Liquid Chromatography with UV detection
HRP	Horseradish Peroxidase
i.v.	intravenous
K	Ketamine
LC	Liquid Chromatography
LC-ESI-MS	Liquid Chromatography-Electrospray Ionisation- Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLE	Liquid-liquid Extraction
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
MAA	Methacrylic Acid
MDA	3,4-Methylenedioxyamphetamine

MDEA	3,4-methylenedioxy-N-ethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MIP	Molecularly Imprinted Polymer
MISPE	Molecularly Imprinted Solid Phase Extraction
NIP	Non-imprinted Polymer
NK	Norketamine
NMDA	*N*-methyl-*D*-aspartate
NSI	Nanospray Ionization
RIA	Radioimmunoassay
RSD	Relative Standard Deviation
SD	Standard deviation
SEM	Scanning Electron Microscopy
SIM	Selected Ion Monitoring
SPE	Solid-Phase Extraction
SPME	Solid-Phase Micro Extraction
SRM	Selected Reaction Monitoring
TMB	3,3',5,5'-tetramethylbenzidine
UV	Ultraviolet

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Norlida Harun,
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1 GENERAL INTRODUCTION

1.1 Forensic Toxicology

A poison is defined as a substance which, taken by any route, can cause a noxious action on the body [1]. Toxicology, or the science of poisons, is often considered to have been founded by Paracelsus, the "father of toxicology", in the 16th century. Toxicology is about the harmful effects of poisons on living organisms subsequent to the chemical and physical properties of the poisons. These include the symptoms, methods of treatment and identification of the poisons.

Forensic toxicology is a subspecialty in toxicology concerned with medico-legal aspects of the harmful effects of poisons [2]. Arguably, the most important aspects of forensic toxicology are the identification of poisons by chemical analysis and subsequent interpretation of the test results and, as a result, the technology and techniques for obtaining and interpreting the results are the main concerns in this field [3].

The criminal justice system increasingly relies on the output of forensic toxicology laboratories to provide evidence relevant to forensic cases, including homicides, sexual assaults and impaired driving, especially when linked to fatalities. Therefore, the identification and quantification of substances of toxicological relevance have become more demanding and challenging.

Forensic toxicology has been developed considerably from its inception to include branches such as post-mortem toxicology, driving under the influence of drugs (DUID), workplace testing, drug abuse, drug facilitated assault, doping in sport and analysis of natural products [4].

1.2 Challenges in Forensic Toxicology

Today new drugs or new derivatives of drugs continue to emerge, especially synthetic drugs such as those in the amphetamines group, and toxicologists have to develop new methods and techniques for their identification. Sensitivity and selectivity are critical in forensic toxicology testing, in which an analytical

technique should be able to detect trace levels of drugs even in the presence of large concentrations of other drugs.

Forensic toxicologists have been able to respond to the challenge of detecting new drugs and metabolites. Toxicologists from all over the world have developed analytical methods for these new drugs. A few recent examples of these were methods developed and validated by researchers for identification in post-mortem blood of 25 opioid drugs [5], 13 amphetamines and derivatives [6], cocaine and its metabolites [7] and 20 anti-depressants [8].

It is impossible to use one extraction procedure to isolate all types of drugs from a sample. In fact, in real casework it is possible to miss four or five drugs that are actually present in a sample. This error is often caused because current methods cannot detect and identify all foreign substances present, or else the concentrations of certain drugs are so low that they are below the detection limits of many techniques. However, in response to this challenge, methods have been published for screening common drugs of abuse using chromatographic techniques [9,10, 11].

There are also challenges in the use of different types of alternative specimens such as oral fluid, hair and vitreous humour in situations when the usual samples such as blood and urine are not available. This gives the toxicologist wider scope for obtaining information and providing evidence but he or she is still limited by the acceptance of the techniques in a court of law, where issues of good laboratory practice and method validation arise because of the lack of internationally-accepted guidelines, as discussed by Cone [12], and lack of accredited laboratories, as found in a survey carried out by the Society of Hair Testing [13] . Recently, a few guidelines have been published that include alternative specimens, for example those produced by the Society of Hair Testing (SOHT) [14] and the Society of Forensic Toxicologists (SOFT) [15].

Recently, legal systems in many countries have recognised more and more the impact of forensic science, including forensic toxicology, in the medico-legal process. One consequence of this growth in prominence is that scientific evidence in general and toxicology results in particular are subject to greater scrutiny than ever before. This situation has triggered further challenges to

toxicologists and has both given opportunities but also demands for them to refine and update toxicology testing in the future.

1.3 Possible Solutions to Current Problems in Forensic Toxicology

One possible solution for the problem of strengthening forensic toxicology testing is by improving the techniques of sample preparation. Sample preparation techniques such as solid phase extraction (SPE) and liquid-liquid extraction (LLE) have been used routinely in forensic laboratories for many years. Solid phase extraction has emerged dramatically to fulfil the need for sample preparation techniques to enhance selectivity and sensitivity.

SPE began in the mid 20th century using adsorbents such as Amberlyte XAD-2 resin, a polystyrene material. Then in the 1980's, substituted silica-based materials used as SPE sorbents began originally through the work of Professor Michael Burke of Arizona University and were put into the market place by the first commercial supplier, Analytichem.

Ten years later mixed-mode sorbents such as Certify™ were introduced and were shown to improve extraction of basic drugs and minimize the number of co-extracted interferences. These were followed a few years later by polymeric sorbents such as Strata™ X which have the advantage of having no residual surface silanol groups, unlike the substituted silica sorbents. Later in 2008, the first molecular imprinted polymer (MIP) based SPE sorbent was introduced [16].

Solid phase micro extraction (SPME) is a miniaturized and solvent-free sample preparation technique that has also been introduced to analytical toxicology. SPME uses a coated silica needle for extraction but is only used routinely in optimised methods for specific analytes in forensic toxicology laboratories [17].

To overcome the issue of sensitivity and selectivity, new products for sample preparation have been introduced commercially or have been synthesised in the toxicologist's own laboratory, such as molecularly imprinted polymers (MIPs) which demonstrate superior selectivity and sensitivity and can minimise matrix effects.

They are in the form of sorbent in cartridges and can be used for typical solid phase extraction (SPE) methods for isolation of analytes from biological matrices. These new products provide the analyst with alternatives to SPE and liquid-liquid extraction (LLE) which for a long time have been used routinely. Molecularly imprinted solid phase extraction (MISPE) was investigated in the work for this thesis.

Chromatography plays a central role in the forensic identification of substances in biological fluids. This has traditionally used gas chromatography with a range of detectors including the flame ionisation detector (GC/FID), the nitrogen-phosphorus detector (GC/NPD) and the mass spectrometer (GC-MS) and/or high performance liquid chromatography with UV detection (HPLC/UV), to detect all the toxicologically-relevant substances present in a subject. However, the change that has been occurring in many forensic laboratories across the world is the implementation of liquid chromatography-tandem mass spectrometry (LC-MS/MS) [18], as demonstrated by the exponential increase in the number of publications per year which was almost double the GC-MS publications even back in 2003.

Recently, the advances in LC-MS/MS systems, especially for detection and quantification of more polar, thermo-labile or low-dose drugs, may have provided the basis for analysis techniques in the future. This equipment is both a good supplement and a good complement to GC-MS for multi-analyte procedures for screening and quantification of drugs in clinical and forensic toxicology as discussed by Maurer [19]. LC-MS/MS may potentially replace immunoassay due to the ease of development of new methods. In areas where immunoassay products are not available and high sensitivity is needed, GC-MS has been used as a screening technique. However, many of the compounds of interest require derivatization to be detectable at appropriate levels.

Compared to GC-MS, LC-MS/MS excels at detecting a wide range of analytes without derivatization. However LC-MS/MS also has disadvantages, such as irreproducibility of fragmentation patterns and reduction of ionization due to matrix effects, as experienced by the present work. The use of LC-MS/MS for

identification and quantification has, together with an enzyme-linked immunosorbent assay (ELISA), been investigated in this thesis.

Urine, whole blood and hair were used for identification of ketamine and its metabolites and drugs in the amphetamines group in the work for this thesis. Urine is used because the method of collection is non-invasive, a large volume of sample can usually be obtained, sample pre-treatment and analysis are simple, there is often a wide drug detection window and parent drug and metabolites are often present in high concentrations compared to other matrices. Furthermore, analytes of interest have been found to be stable in urine for a longer time if the samples were frozen [20,21]. However, the analyst must be aware that urine samples can be adulterated by substances such as detergent, vinegar and table salt to give false negative results [22].

Blood samples are used because most toxic substances can be detected in a blood sample and they have been found very useful in forensic toxicology as their concentrations may be interpreted, within limits, in terms of the effects on the subject. Hair samples are a good way to test for substance abuse that has occurred over the long term as well as being non-invasive and not easily adulterated.

1.4 Method Validation

For the results of toxicology testing to be scientifically valid in terms of accuracy, reliability and adequate for intended purposes such as to be presentable in court of law, the methods and procedures used for analyzing specimens must be validated. Method validation procedures used in the work for this thesis were dependent on whether the method concerned was qualitative, for example, immunoassay (ELISA) or quantitative, for example LC-MS/MS. Not all validation parameters are appropriate for each type of method and these are discussed below.

1.4.1 Method Validation for Immunoassay

Three important parameters for validation of immunoassay are selectivity/ cross-reactivity, limit of detection (LOD) and precision [23].

Selectivity/cross-reactivity is tested by analysing samples under specified test conditions which have been spiked with metabolites of the target analyte, drugs in the same group and other classes of drugs/subtances commonly found in the matrix in which the drugs is analysed. The assay should also be tested with samples known to be positive or negative. A very selective immunoassay would have no significant cross-reactivity with other drugs or substances.

The LOD is obtained by measuring the absorbance of 10 blank samples which have been spiked with different concentrations of the target analyte to determine the minimum concentration which can reliably be detected. The other way to obtain the LOD for an immunoassay kit is by measuring the absorbance of 10 blank specimens and calculating values for the mean and standard deviation of these readings. The concentration corresponding to the mean absorbance value of the blanks minus 3 standard deviations is obtained from the immunoassay calibration curve and this approximates to the LOD. The LOD value should be substantially lower than the cut-off concentration to discriminate between negative and positive samples.

The intra-day precision was determined by analysing 10 blank samples spiked with the analyte of interest at a concentration equal to the cut-off value on the same plate and on the same day. For inter-day precision the tests are carried out on five different days with different batches of reagent kits. The RSD values for both should be better than ±20%.

Immunoassay validation may also include characterisation of the calibration model (the dose-response curve) which is the ability of the test method to produce test results which are proportional to the concentration of the analyte in the sample within the expected concentration range. A range of concentration levels of analyte of interest is spiked into blank samples and a graph of the dose response curve is plotted. A cut-off value of the assay is selected from the linear part of the response [24].

1.4.2 Method Validation for LC-MS/MS

The components of method validation tested for LC-MS/MS in the present work were linearity, limit of detection (LOD), lower limit of quantification (LLOQ), intra-day and inter-day precision, accuracy, recovery and matrix effects. The procedures for the method validation were conducted based on guidelines from United Nations Office on Drugs and Crime (UNODC) on the Implementation of a Quality Management System in Drug Testing Laboratories [25] and from the book by Bliesner on the validation of chromatographic methods [26].

1.4.2.1 Linearity

Linearity is the ability of a test method to produce test results which are proportional to the concentration of the analyte in the sample within the expected concentration range. The expected concentration should depend on the concentrations of target analytes found in the body. Linearity is evaluated using a minimum 6 point standard calibration curve within a concentration span of 80 % - 120 % of the expected range and should be established by visual inspection of the analytical response [27,28].

In this study, the linear response relationship between the peak area ratio of an analytes and its internal standard and concentration has been evaluated statistically from the calculation of the regression line by the method of least squares, which gives the slope of the line, the intercept on the y axis and the correlation coefficient (R^2). The good linearity means there is no significant deviation from linearity over the concentration range, which means a correlation coefficient of 0.99 or better is required for a good method of analysis [15,25].

1.4.2.2 Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The limit of detection (LOD) of an analytical procedure is the lowest concentration of an analyte in a sample that can be detected by a method but not necessarily quantified as an exact value. The lower limit of quantification (LLOQ) is defined as the lowest concentration in the sample that can quantitatively determined with acceptable precision and accuracy.

There is more than one procedure to obtain the LOD and LLOQ values. In the most used method the LOD and LLOQ are determined by the value of the signal to noise (S/N) ratios of 3 and 10 of the analyte peak to matrix background noise [28]. The LLOQ also can be defined as the lowest calibration standard that can be measured with acceptable precision and accuracy [15,28].

In the work of this study, the LOD and LLOQ were calculated statistically from the regression line of the calibration curve. LOD was calculated using 3 times the standard error of the regression line and the LLOQ using 10 times the standard error of the regression line. This type of calculation was found to be easier and fit for the purpose of method validation in this study and has been used in method validation previously [29,30].

1.4.2.3 Recovery

Recovery is a measure of the efficiency of a method in isolating analytes from their matrix and is reported as the percentage of the known analytes of interest which are obtained from a complete method, including the extraction and analysis. Recovery can be determined by comparing the response of an extracted standard with that of the neat, unextracted solution which is assumed to give 100 % recovery. The corresponding internal standard is added after the extraction to allow direct comparison with the unextracted standards. The experiment was carried out using at least five replicates of low, medium and high concentration levels in the expected range. The acceptable recoveries in an analytical method are usually more than 50 % [28].

1.4.2.4 Selectivity (Specificity)

Specificity refers to the ability of a method to produce a response of single analytes only while selectivity refers to the ability of the method to distinguish the response of a single analyte from other analytes in the complex mixture. Selectivity is the most important component in the validation of a chromatographic method. Selectivity is obtained by optimising separation and detection of standard mixtures and also by observing the selectivity of the method in the analysis of real samples.

It is suggested that selectivity and specificity are investigated by analysing six different sources of blank specimens. However, in the work of this study an LC-

MS/MS was used and it is sufficient to include a blank (negative sample) from the same matrix in every batch analysis of case sample to assess the presence of possible interfering analytes that will affect the selectivity of the target analytes [28].

1.4.2.5 Precision and Accuracy

Precision is the closeness of agreement (degree of scatter) between a series of measurements obtained under a set of prescribed conditions. Precision is expressed as percentage of the coefficient of variation (CV %) or percentage of relative standard deviation (RSD %).

In the work of this study, repeatability or intra-day precision was measured by analysing five replicates of the specimen on the same day, using the same method at low, medium and high concentrations. Reproducibility or inter-day precision was measured in the same manner over five different days. The method should show a good consistency for all replicates and a % RSD of 20 % or less is considered to be acceptable for a valid method [15].

Normally, accuracy or the degree of closeness to the determined value was assessed together with the precision study. The experiment was carried out using five replicate blank samples spiked at low, medium and high concentrations in the expected calibration range. A calibration curve was plotted and the accuracy was determined by comparing the mean calculated concentration of the spiked urine samples with the target concentration. The acceptable limit was the same as for precision with an % RSD of 20 % or less considered to be acceptable [15].

1.4.2.6 Matrix Effects Assessment

Matrix effects can be defined as the change in response observed for a given concentration of target analyte in the presence of other sample components. The presence of matrix components in biological fluids can cause suppression (under-estimation) or enhancement (over-estimation) of the target analyte response. As demand for assay sensitivity requirements increases, the need for improved sample preparation methods becomes critical in order to limit matrix effects.

Electrospray ionization (ESI), which was used in this study, can be affected by factors other than target analyte concentration more than atmospheric pressure chemical ionization (APCI) [31] – and this can compromise or invalidate data. Therefore matrix effect assessment becomes compulsory and was included in this study. Matrix effects could be reduced (clean up) or magnified (pre-concentrate) by sample preparation steps such as extraction, as demonstrated by previous studies using different types of sample preparation [31,32].

The matrix effect assessment in this study was determined following the method of Matuszewski et. al [33]. Replicate blank samples from different individuals were collected to obtain accurate data on the size of matrix effects between individuals and to evaluate the robustness of the matrix effect analysis.

The blank samples were spiked at low, medium and high concentrations and compared with other replicates which were spiked with the same concentrations in the loading solvent (neat solutions). Internal standard was added after the extraction. The percentage matrix effect was the peak area ratio of the analyte to internal standard in human matrix (urine, blood, hair extract) over the peak area ratio of the analyte to internal standard in the neat solution, respectively.

1.5 Conclusion

In conclusion, the toxicologist must be up-to-date with respect to technology and techniques for obtaining and interpreting analytical results, with more attention being given to sensitivity and selectivity in the validation of methods in the laboratory. Adequate training must be provided on new equipment and techniques, on proper method validation and laboratory accreditation as well as professional training in serving as an expert witness in court.

Toxicologists also must be able to use all sorts of opportunities such as the availability of different sample matrices and the advance in new equipment such as LC-MS/MS for improvement of the investigation methods in the laboratory. Furthermore, validation of methods and techniques provides more confidence for their application to routine and research work.

In response to the challenges in the field, the work in this thesis investigated the use in forensic toxicology of one of the latest techniques in sample preparation, molecularly imprinted polymer solid phase extraction (MISPE) together with LC-MS/MS for detection and quantification of drugs. It was hoped that data collected from this work will be useful and provide some information on the techniques available for forensic toxicology testing.

1.6 Aims of the Study

This study involved the development and validation of both screening and confirmatory detection methods for ketamine and its metabolites in biological samples. Various extraction methods were applied prior to analysis and identification of these compounds with a mass spectrometry technique. The aims of this project could therefore be summarized as follows:

[1] Development and validation of an ELISA screening method for ketamine and its metabolites.

[2] Development and validation of an LC-ESI-MS/MS method for ketamine and its metabolites in urine.

[3] Investigation of the use of SPE and MISPE extraction techniques for ketamine and its metabolites in hair.

[4] Comparison of MISPE and SPE methods for the detection and quantification of ketamine from hair samples by LC-ESI-MS/MS.

[5] Evaluation and validation of MISPE for amphetamines in whole blood.

2 KETAMINE

2.1 Introduction

Ketamine is not a new drug but has been used for over 40 years. It is a rapidly acting dissociative anaesthetic which was first synthesised in 1962 by Calvin Stevens at Parke-Davis laboratories, Michigan [34] .The drug was patented in 1963 in Belgium and in 1989 in the United States of America (USA). It was first marketed in the early 1970s as an alternative to phencyclidine [35], which produced side effects such as hallucinations and delirium. A pharmacological effect common to both ketamine and PCP is the separation of perception from sensation and this is the reason that they are members of the same drug group [36].

Ketamine has been used clinically as an anaesthetic in veterinary medicine and to a lesser extent in human medicine, especially for emergency treatment. It has three important drug properties: it is a hypnotic (sleep inducing), analgesic (pain relieving) and amnesic (short term memory loss).

In the United Kingdom, ketamine is classified as a Class C drug under the Misuse of Drugs Act 1971, which carries up to two years imprisonment and/or an unlimited fine for possession and up to fourteen years imprisonment and/or an unlimited fine for possession to supply or supplying the drug [37]. Ketamine is in the same class as anabolic steroids, benzodiazepines and gamma-hydroxybutyrate (GHB).

Recreational use of ketamine was first reported in the United Kingdom and European countries in the 1990s. Ketamine has been classified as one of the club drugs by the National Institute on Drug Abuse (NIDA) in the USA because it was being abused by an increasing number of young people in clubs and was being distributed at raves and parties [38]. The trend of ketamine abuse has been followed in Asian countries, especially China, Taiwan and Singapore [39].

2.2 Chemical and Physical Properties of Ketamine

Ketamine is an arylcycloamine and in systematic nomenclature it is 2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone, $C_{13}H_{16}ClNO$, molecular weight 237.74. It is a weakly basic, lipid soluble compound which contains a chiral centre at C2 of the cyclohexane ring, forming two enantiomers, *S-(+)-ketamine* and *R-(-)-ketamine*. The molecular structures of ketamine enantiomers are shown in Figure 2-1. The hydrochloride salt is soluble in water, methanol and chloroform and a solution of ketamine. The racemic HCl standard in methanol is available commercially as an analytical standard. The melting points of the free base and hydrochloride are 92-93°C and 262-263°C respectively.

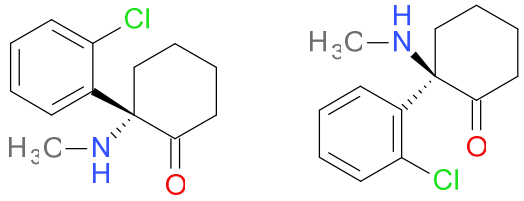


Figure 2-1 Molecular structures of S-(+)- ketamine (left) and R-(-)- ketamine (right)

Ketamine manufactured by pharmaceutical companies must meet the standards of Good Manufacturing Practice (GMP) which means purity and quality are guaranteed. The racemic form is used for pharmaceutical purposes as the hydrocholoride salt, prepared in aqueous solution and packaged in small glass vials, ready for parenteral administration. It is marketed as Ketalar®, Ketolar®, Ketaject®, Ketanest®, Ketavet®, Ketavet 100®, Ketalin®, Vetalar®, Kalipsol®, Calipsol®, Substania®, Ketamine Panpharma®, Ketamine UVA®, Chlorketam® and Imalgene®.

On the street ketamine is commonly known as K, Ket, Special K, Vitamin K, Kit Kat, Keller, Kelly's Day, Green, Blind Squid, Cat Valium, Purple, Special La Coke, Super Acid and Super C [40]. It is available in powder form, tablet and as a vaporiser of unknown purity and quality.

2.3 Pharmacokinetics

The reported volume of distribution varied from 1.5 to 3.21 l/kg. The clearance was in the range of 12-28 ml (kg.min). Volume of distribution and clearance for the S-(+)-ketamine are 9 to 14 % greater than the R-(-)-ketamine, respectively [41].

2.3.1 Absorption

In the clinical setting the most common route of administration into the body of ketamine in solution is by injecting intramuscularly. It is more rapidly absorbed (T_{max} 5-15 min) compared to the nasal (T_{max} 20 min) and oral (T_{max} 30 min) routes. The bioavailability decreases from intramuscular to oral or rectal administration by 17 % or 25 % respectively, because of the effects of first-pass metabolism in the liver and intestine. It is even lower after nasal administration (the bioavailability is less than 50 % of that obtained by intramuscular administration)[42]. The reason is unknown but it is assumed to be caused by swallowing of a significant amount of the intranasal deposit.

2.3.2 Distribution

Ketamine base has a high lipid solubility and low plasma binding (12 %). It readily crosses the placenta and rapidly transfers across the blood-brain barrier, having immediate effects on the subject, whether human or animal. Studies in animals showed it was highly concentrated in lung, body fat and liver while lower concentrations were obtained in heart, skeletal muscle and blood plasma. After absorption, ketamine is distributed to highly perfused tissues such as brain (the distribution phase half-life after i.v. administration was 24 sec) and achieves four to fives times higher concentration than in the plasma. Then, CNS effects subside, following redistribution to less well-perfused tissues (re-distribution half-life 2.7 min).

2.3.3 Biotransformation

Phase I biotransformation of ketamine primarily takes place in the liver. Ketamine undergoes extensive hepatic metabolism with only 2 % of the dose

recovered unchanged in the urine. 88 % of the dose can be recovered as metabolites in the urine over 72 h. Ketamine undergoes N-demethylation by liver microsomal cytochrome P450 enzymes CYP3A4, CYP2B6 and CYP2C9 to form its primary metabolite, norketamine (metabolite I) [43].

Norketamine is hydroxylated on the cyclohexanone ring at positions 5 and 6 to form metabolite III and IV which can be excreted as conjugates or converted to metabolite II (dehydronorketamine) by dehydration. Ketamine itself can undergo hydroxylation on the cyclohexanone ring at positions 5 and 6 to form metabolites V and VI which can similarly be excreted as conjugates or converted to metabolite VII by dehydration.

Metabolite VII can be transformed to metabolite II (dehydronorketamine) by N-demethylation. Only 16 % of the dose can be recovered in urine as hydroxylated ketamine or norketamine. Phase II involves metabolism of hydroxylated metabolites results in formation of O-glucuronides which are water soluble and are easily excreted in urine (> 80% of the dose).

The main metabolites, norketamine and dehydroxynorketamine can be measured in the same assay as for ketamine due to their closely related chemical structures. The metabolism route of ketamine is depicted in Figure 2-2 [44,45].

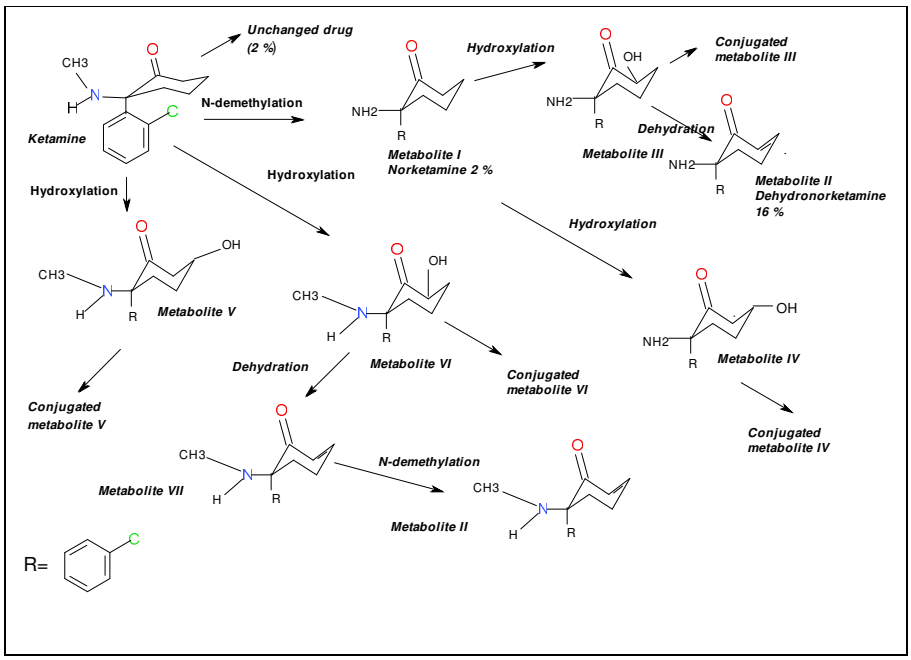


Figure 2-2 Metabolism of ketamine

When taken orally or rectally, the initial plasma concentration of norketamine is higher than ketamine but the plasma area under the curve is similar for all routes of administration. The bioavailability of ketamine is low when given orally or rectally due to extensive first pass metabolism in the liver and intestine which is largely responsible for this effect [46]. Norketamine has the analgesic properties of ketamine but has only one third of its anaesthetic potency. Dehydronorketamine (DHNK) was originally believed to be an analytical artefact caused by the high temperature of gas chromatography but recent research findings have shown that DHNK is a true metabolite of ketamine [47]. The anaesthetic effect of DHNK is unknown.

2.3.4 Elimination

The main route of ketamine elimination is by liver metabolism to hydroxy metabolites which are conjugated and mainly excreted via the kidney in the urine. Ketamine and norketamine can be detected in urine up to 5 and 6 days after administration, respectively, while dehydronorketamine can be detected in urine for up to 10 days [48]. Renal elimination accounts for 90 % of the total with about 4 % of unchanged ketamine, while fecal elimination accounts for up to 5 %. Ketamine clearance was susceptible to blood flow-affecting factors due to its high extraction (0.9). The elimination half-life for ketamine is in the range of 2 to 3 hours.

2.4 Pharmacodynamics

Ketamine is a dissociative anaesthetic drug. The term “dissociative” refers to a psychological state in which the user experiences a feeling of dissociation of the mind from the body [49,50]. The pharmacology of ketamine involves the effects of the substance on the neurotransmitter systems related to its clinical and recreational use (primary pharmacology) and also the effects of this drug on various organs in the body (secondary pharmacology).

2.4.1 Effects on the Central Nervous System (CNS)

Ketamine appears to exert most or all of its effects in the Central Nervous System (CNS). It binds to the PCP-binding site which is located within the cation

channel of neurones and a separate site of the N-methyl-D-aspartic acid (NMDA) receptor complex. NMDA receptors function as calcium-gated ion channel receptors. Binding of ketamine blocks the transmembrane ion flux and ketamine acts as a non-competitive NMDA-receptor antagonist.

The activation of the endogenous NMDA receptors by excitatory amino acids, such as glutamic acid, aspartic acid and glycine results in opening of the ion channel and depolarisation of the neurone. They are involved in sensory input at the spinal, thalamic, limbic and cortical levels. Ketamine would be expected to block sensory input to the higher centres of the CNS (NMDA receptors) and disturb the processing of memory, thinking and learning [51] .

Some effects of ketamine may be caused by its action on the catecholamine system which notably enhances dopamine activity [52,53]. Ketamine increases NAc dopamine efflux by mobilization of the dopamine storage pool to release sites and not by blocking dopamine reuptake, auto receptors or NMDA receptors [54].

Repeated ketamine administration diminishes the initial five-fold increase in dopamine release in the prefrontal cortex, while enhancing the level of extracellular 5-hydroxyindole acetic acid (5-HIAA), a serotonin metabolite. Repeated ketamine administration can therefore alter the balance between dopamine and serotonin neurotransmission in the prefrontal cortex [55]. This suggests that the addictive, euphorigenic and psychotomimetic properties of ketamine may be related to the dopaminergic effects.

S-ketamine has a 3 to 4 fold higher affinity for the PCP site of the NMDA-receptor than R-ketamine and therefore has more anaesthetic potency. It is also known that S-ketamine is the enantiomer that causes the psycotomimetic effects of ketamine, although sub-anaesthetic doses of the R-ketamine may induce a state of relaxation [41,52].

Several studies have indicated the relationship between opioid receptors and the pharmacological effects of ketamine [56]. Crisp et al. reported that the analgesic effects of ketamine can largely be attributed to the action of these spinal and central receptors [57]. A more recent study in rats by Ji et al. indicated that ketamine has the ability to suppress morphine withdrawal syndrome in an

experimental setting without motor interference and Nucleas Accumbens is mentioned as the site of action [58].

The major metabolite norketamine (NK) is pharmacologically active but has a shorter duration of action. It has one third of the anaesthetic potency and binding affinity to the NMDA-receptor compared to the parent compound. Norketamine mainly contributes to the analgesic properties of ketamine [59] and also contributes to the antinociceptive effect of ketamine [60].

2.4.2 Effects on the Cardiovascular System

Ketamine appears to stimulate the cardiovascular system and can produce changes in heart rate, blood pressure and cardiac output as reported by Haas and Harper [61] . The cardiovascular side effects of ketamine use include hypertension and sinus tachycardia, hypotension and sinus bradycardia and other cardiac arrhythmias. Patients with high blood pressure, ischemic heart disease or cerebrovascular accident must avoid the use of ketamine but its use is not a problem to a healthy individual. Following recreational use, physical examination showed that most of the patients brought into the emergency and trauma department of a hospital were diagnosed with the symptom of tachycardia [62].

2.4.3 Effects on the Respiratory System

Following high doses of ketamine, respiratory depression and apnea can occur. Respiratory depression was reported in some cases after rapid intravenous injection and also after routine paediatric use of ketamine administered intramuscularly [63,64]. Laryngospasm and other forms of airway obstruction may occur in rare cases. This does not happen after administration of low doses of ketamine because it is known as a mild respiratory depressant. There have been no cases of respiratory depression reported as a result of recreational use of ketamine. High doses of ketamine cause a shift of the CO_2 dose-response curve to the right but do not change the slope. Therefore, the respiratory drive in response to CO_2 is depressed by 15 to 22 %. Other effects of ketamine include increases in blood glucose, plasma cortisol, prolactin and muscle tone [65]. Ketamine also decreases intraocular pressure [63].

2.4.4 Common Effects of Ketamine –Non fatal Intoxications

The effects of ketamine use are dose dependent and are affected by the route of administration, body weight and the user's health, thus the duration and severity of effects varies from one person to another. The onset of effects is rapid and occurs a few minutes after administration.

The general effects of ketamine on recreational users include symptoms such as numbness, loss of coordination, sense of vulnerability, muscle rigidity, violent behaviour, slurred or blocked speech, exaggerated sense of strength, blank stare, amnesia, agitation, memory loss, unconsciousness, nausea and delerium. The predominant symptom on physical examination for cases brought to emergency department was tachycardia followed by rhabdomyolysis [62].

The main effects include neuro-behavioural, anxiety (especially for first-time users), agitation, change of perception (loss of notion of danger, visual disturbances), disorientation and impairment of motor function, such as ataxia, dystonic reaction. To some extent, users also cannot feel pain and this can lead to self injury [66-68] .

A team from the University College London who carried out a range of memory and psychological tests on 120 frequent ketamine users found that ketamine harms memory. This fact was supported by poor performance on skills such as recalling names, conversations and patterns [69]. The relationships between the route of ketamine administration, the dosage, the onset, the effects and duration of action are shown in Table 2-1.

Table 2-1 Common effects of ketamine

Administration	Dosage	Onset	Effects	Duration
Intramuscular injection	10-40 mg 60+ mg	3-4 minutes after injection	Mild hallucinations Out of body, near death hallucinations; terrors	45-90 minutes
Intranasal ingestion	10-60 mg 100+ mg	5-15 minutes after ingestion	Mild hallucinations Out of body, near death hallucinations; terrors	10-30 minutes
Oral ingestion	40-75 mg 200+ mg	5-20 minutes after ingestion	Mild hallucinations Out of body, near death hallucinations; terrors	Up to 90 minutes

2.5 Toxicological Data

The non-human single acute dose toxicity (LD_{50}) of ketamine is 140 mg/kg intra-peritoneal in the neonatal rat and 616 mg/kg body weight orally in the mouse [70] while the LD_{50} values in adult mice and rats are 224 mg/kg and 229 mg/kg, respectively. In the human it was estimated that intravenous doses of more than 11.3 mg/kg can be fatal, which is equivalent to a dose above 680 mg for a 60 kg human. A summary of fatal cases involving ketamine is shown in Table 2.2. In most of these cases ketamine was taken in combination with other drugs such as benzodiazepines, cocaine, opiates, alcohol and other anaesthetics.

4 out of the all cases in the table identified ketamine as the only source of the fatal intoxication, while the other cases involved mixed drug fatalities or a minor role for ketamine. The ketamine concentrations in these cases were in the anaesthetic range or above, 4-17 times the recommended dose for anaesthesia; or 1.3-2.5 times the recommended intramuscular dose for anaesthesia (based on a body weight of 60 kg) [71].

Table 2-2 Fatalities involving ketamine

Case number	Country	Sex	Age	Drugs involved	Drug concentration		
					Blood µg/ml	Urine µg/ml	Other µg/g
1	Italy	M	18	Ketamine Norketamine	27.4 present, not quantified	8.51	bile=15.2 brain=3.2 liver=6.6 kidney=3.4
2	USA	F	31	Ketamine Norketamine	- present, not quantified	- -	liver=6.3 kidney=3.2 lung=1.6 heart=2.4
3	USA	M	46	Ketamine Norketamine	3 not present	- -	liver=0.8 kidney=0.6 spleen=0.8 brain=4.0 lung=2.2 heart=3.5
4	Italy	M	34	Ketamine Norketamine	- -	- -	liver=12.2 brain=3.7 lung=16.4 kidney=15.5 spleen=14.1 liver=0.15 brain=4.0 lung=0.17 kidney=0.7 spleen=0.12
5	USA	M	45	Ketamine Tiletamine Zolazepam	0.037 0.295 1.71	0.031 0.682 1.33	- liver=0.196 liver=15.5
6	USA	M	32	Ketamine Ethanol Nalbuphine	1.8 170 <0.020	2 - -	Brain=4.3 spleen=6.1 liver=4.9 kidney=3.6
	France	M	-	Ketamine Norketamine chloroform cocaine benzoylecgonine methylecgonine ester anhydroecgonine ester codeine 6-MAM thiopental pentobarbital diazepam nordiazepam oxazepam	1.4 - 30.7 0.576 - 0.78 0.12 0.013 0.420 1.97 2.64 0.070 0.2 -	present present present present present present present present - - - - present	hair =11.3 hair=1.0 - hair=5.5 hair=1.5 hair=1.0 - - hair=4.4 hair=3.4 hair=5.3 hair=10.0 hair=1.2 hair=0.1

Lalonde and Wallage [72] reported 2 fatality cases which were attributed to ketamine use in Canada. The concentration of ketamine in heart blood in these cases was 6.9 and 1.6 mg/L, respectively, while the concentration of ketamine in femoral blood was 1.8 and 0.6 concentrations mg/L, respectively. The first case strongly pointed to ketamine as the sole factor of the fatal poisoning, and the other case involved asthma as a co-morbidity factor in the death.

In Taiwan Lin et al. reported 59 fatalities involving methylenedioxy-methamphetamine (MDMA) use in the years 2001 to 2008. Of these, 37 were attributed to drug intoxication and 22 to trauma, with drugs being an incidental finding. Within the group attributed to drug intoxication, 16 fatalities were caused by MDMA alone, with concentrations from 0.48 µg/mL to 16.53 µg/mL in post-mortem blood. Another 15 fatalities in this group involved concurrent use of MDMA with ketamine and other drugs, including lidocaine, chlorpheniramine, morphine, flurazepam, phenytoin and ethanol. The other 6 fatalities were intoxications involving MDMA and multiple drugs other than ketamine [73]. Earlier in 2000, an article by Gill and Stajic in New York City reported 87 ketamine-positive deaths over a 2 year period (1997-1999) reviewed by the New York City Chief Medical Examiner. 1 out of 15 non-hospital deaths was a fatal intoxication exclusively involving ketamine, 12 were caused by acute multi-drug intoxications with opiates, amphetamines and cocaine, 1 involved ethanol and 1 had an unknown cause [74].

2.6 Medicinal Use of Ketamine

Ketamine is used in humans as an anaesthetic in combination or alone, for short term paediatric and adult surgical procedures, especially in emergency and trauma care. The major drawback after awakening from ketamine anaesthesia is the occurrence of emergence reactions such as hallucinations, vivid dreams, floating sensations and delirium. These emergence phenomena appear to occur more frequently in adults (30-50 %) than in children (5-15 %) [51,64] . For this reason, it is usually used concurrently with benzodiazepines to put the patients in a low stimulus environment.

For clinical analgesic purposes, it is administered intramuscularly, intravenously, orally, rectally and intrathecally. An intravenous dose equivalent to 2 mg of

ketamine per kg body weight (ranging from 1.4 - 4.5 mg/kg) given in 60 seconds produces surgical anaesthesia lasting 5 to 10 minutes while an intramuscular dose equivalent to 10 mg per kg body weight (ranging from 6.5 - 13 mg/kg) produces anaesthesia within 3 to 4 minutes and lasting for 12 to 25 minutes. The i.v. and intramuscular doses for children are 0.5 - 2.0 mg/kg and 3 - 7 mg/kg, respectively.

Analgesic effects are produced by a dose of 0.2-0.75 mg/kg intravenously and 0.5 mg/kg intramuscularly or orally. In the liver, ketamine is transformed to norketamine, the major metabolite. The analgesic effect was similar through different routes of administration due to the strong analgesic properties of norketamine which is also an NMDA receptor inhibitor and has a half maximal inhibitory concentration (IC_{50}) about 10 times higher than that of ketamine [75,76]. Psychotropic effects of ketamine are produced with a dose ranging from 25 - 200 mg/kg intramuscularly and 0.1 - 1.0 mg/kg intravenously.

2.7 Misuse of Ketamine

Ketamine is available illicitly in liquid, powder and pill forms. It can be taken orally, smoked, snorted, drunk or injected. It is believed that legitimate ketamine is obtained in bulk from pharmaceutical companies in the USA and United Kingdom and then distributed worldwide for black market purposes. It is known that the synthesis of ketamine is too difficult for a clandestine chemist and because of that they use legitimate sources and mix the ketamine with other drugs such as amphetamines and benzodiazepines.

Ketamine misuse was first spotted at the West Coast of USA in the early 1970s [77]. Recently, a survey in the USA reported that ketamine is commonly injected intramuscularly among the youth, involving multiple injections, shared bottles of ketamine and used syringes from secondary sources. These practices increase the risk of transmitting infectious diseases such as HIV and Hepatitis C and are different from those used with other drugs such as heroin, cocaine or methamphetamine, which are injected intravenously and or once or twice in a session by users alone or in small groups [78].

There have been several reports on the recreational use of ketamine in UK in the early 1990s [79,66]. In 2005, the DrugScope Street Drug Trends Survey first highlighted the emergence of ketamine use as a recreational drug. The finding indicated that the drug had become a major street drug in eight of the 15 UK towns and cities surveyed, in which nine of the 20 areas surveyed in the 2008 research reported an increase in ketamine use [80]. Furthermore, recreational use of ketamine has also been reported in EU countries [67] as reported by the EMCDDA inquiry, 2002.

Lifetime use of ketamine was reported by 1 % of Australians aged 14 years or older, with 0.3 % reporting recent use. Prevalence of ketamine use was highest amongst those aged 20–29 years who were also more likely to report the recent use of a wide range of other drugs [81].

The recreational use of ketamine at raves, parties and nightclubs has grown, thus increasing public concerns about the potential hazards of this drug [82]. A recent Taiwanese study reported that ketamine was detected in 47 % of the urine samples obtained from a study of those attending rave parties but only in 2 % of the urine samples of police detainees [34]. A recently conducted review of drug use in China mentions that drugs such as amphetamines and ketamine have been abused in the country since 1997, quoting a 2002 survey of poly drug use among heroin addicts in which 16.2% reported ketamine use, and concluded that club drug use was a serious concern in some regions of the country [83]. Ketamine abuse has also been reported in Singapore [39] and most recently has been found in urine samples from suspected drug users in Malaysia [84].

Apart from mainstream recreational use, several smaller groups have been identified in which ketamine is abused, such as in a subset of gay men for sexual pleasure purposes [85], also by homeless and runaway youths which increased rate of risks with regards to sexual use and injecting behaviour of this drug in the USA [78,86].

The extent of crime involved in trafficking, distribution and illicit production of ketamine is not well known and global seizures of ketamine are still low compared to other regularly abused drugs such as amphetamine type stimulants.

These activities are focused primarily in Asia and in Oceania countries, which may be related to the tourism industry in which increasing numbers of European and American tourists are able to get the product in these countries.

In the Annual Reports Questionnaire 2003 submitted by governments to UNDOC, Australia, Greece, Hong Kong, Macao, Philippines and Malaysia reported ketamine seizures; Malaysia and Hong Kong reported the largest seizures (82.5 kg and 51 kg respectively). Then, in the Annual Reports Questionnaire 2004, 4 governments reported seizures of ketamine (Australia, Belgium, Hong Kong and Macao) the largest seizure being reported by Hong Kong (46.44 kg). The amount of ketamine seized each year by the Royal Police of Malaysia from 1998 to 2007 is shown in Table 2.3.

Table 2-3 Ketamine seizures in Malaysia from 1998 to 2007 [87]

Year	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
Ketamine (kg)	0.3	0.8	1.0	7.3	10.0	82.5	11.7	395.9	190.4	177.91

2.8 Ketamine and Drug-facilitated Sexual Assault

Ketamine is known as a drug with dissociative and sedative properties and has been used in drug-facilitated sexual assault (DFSA). In this kind of crime, the drug is mixed in the victim's drink without their knowledge and the victim will be in a state of unconsciousness. Concerned with the harmful effects of these DFSA drugs, a company has came out with a field test kit for detecting ketamine and GHB in beverages called Drink Safe Technology Version 1.2 [88].

There have been reports in the literature about the association of drugs such as flunitrazepam (Rohypnol®), gammahydroxy-butyrate (GHB) and ketamine with DFSA crimes [89,90,91]. There are also government web-sites regarding drug-facilitated sexual assaults, for example, the UK Home Office [92] and West Virginia Foundation for Rape Information and Services [93].

In the UK, ketamine was found in 3 cases of alleged DFSA over a period of 3 years (2000 – 2003) in samples analysed by the Forensic Science Service, London laboratory. 1 case indicated ketamine as the sole drug in the sample, 1 case detected a combination of ketamine and cocaine and the third was a mixture of cannabis, amphetamine, cocaine and ketamine [94].

Strict laws have been enacted with regards to DFSA drugs, for example, ketamine has been included in the Drug-Induced Rape Prevention and Punishment Act 1996 in the USA and an offender may face up to 20 years imprisonment [95].

3 METHODS OF KETAMINE ANALYSIS IN FORENSIC TOXICOLOGY

There are a number of analytical procedures for the detection and identification of ketamine and its metabolites in biological fluids. The specific methods chosen by a laboratory depend on a number of factors such as workload, cost, and availability of instrumentation, turnaround time, sensitivity, specificity and reliability. This chapter will present a review of the methods based on the available literature and the possibility of these methods being used for the work of the present study. The scope of the review includes screening and confirmatory methods.

3.1 Screening Methods

Most screening methods involve immunoassay techniques. Immunoassays are based on the principle of competition between labelled and unlabelled antigen (drug) for binding sites on specific antibody. There are many types of immunoassay employed for analysis of drugs in body fluids such as enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) and radioimmunoassay (RIA). Nowadays, cloned enzyme donor immunoassay (CEDIA) and enzyme linked immunosorbent assay (ELISA) are increasingly popular for drug screening.

The major differences between these immunoassays are in the type of end point indicator used and the sensitivity required. The exquisite sensitivity and the remarkable specificity of immunoassay led to its early application for the detection of drug abuse. The small amount of sample needed for the analysis in immunoassay techniques, for example a few microlitres of urine or a blood, saliva or semen stain, gives a significant advantage to this technique in forensic toxicology applications [96].

Very few papers have previously been published regarding the use of immunoassay techniques for ketamine detection due to the unavailability of commercial test kits. In 2003, there was one paper presented regarding the use of ELISA kit for ketamine screening [97]. Later in 2005, 1 article has been published involving the use of a commercial ELISA kit to detect ketamine and

norketamine in monkey urine [98]. Both studies used the Neogen ELISA kit as the screening kit which can easily detect ketamine and norketamine at 25 ng/mL for screening purposes.

In 2007 a paper was published which reported a comparison between Neogen® and another commercial kit from International Diagnostic System (IDS®) [99] . The comparison is shown in Table 3-1.

Table 3-1 Comparison of 2 commercial kits for ketamine screening in urine

Reagent Characteristics	Neogen®	International Diagnostic System (IDS®)
Cross-reactivity	directed towards ketamine	directed towards ketamine metabolites norketamine and dehydronorketamine
Linear range	narrower compared to IDS ®	wider linear range than Neogen®
Detection limit	higher concentration than IDS ®	lower concentration than Neogen®
Sample analysis	negative for specimen collected at late metabolic stage	positive for specimen collected at late metabolic stage

The study used 100 ng/mL concentrations as the cut off point to differentiate between positive and negative samples prior to a GC-MS confirmation method. In this particular study the authors did not expect a close correlation between ELISA and the GC-MS confirmation method (R^2 < 0.5). This is because ELISA is responsive to compounds structurally related to ketamine while GC-MS is very specific to the analyte, ketamine.

However, until now, no ketamine immunoassay kits for other types of biological samples such as blood, hair and saliva were available on the market. ELISA for ketamine in these biological samples was evaluated and validated in the work for this thesis and the principle of the test will be explained in Chapter 4.

3.2 Confirmatory Methods

Antibodies in the immunoassay kits can react with compounds of related structure and so may lack specificity. Therefore, immunoassay techniques need to be confirmed with another method such as one involving a chromatographic technique to give the definitive results needed in forensic toxicology. Not all confirmatory methods were used in the present work and three main methods, GC-MS, LC-MS and HPLC, will be discussed in this chapter.

Detection of ketamine and its metabolites in biological fluids requires sensitive and reliable methods due to the use of ketamine as an anaesthetic drug in humans and to the possibility of self injury or death caused by misuse of the drug. To date, chromatographic techniques are suitable for the analysis of a very wide range of organic compounds and are often preferred as the analytical methods of detection.

Chromatography involves both preparative (extraction, purification) and analytical (separation, identification, quantification) methods using techniques such as HPLC, GC-MS or LC-MS [100]. Chromatographic analysis can provide qualitative information in terms of characteristic retention parameters, and can give precise quantitative information in terms of peak areas or peak heights, especially when coupled to mass spectrometry.

3.2.1 Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography (GC) is a separation technique in which the mobile phase is a gas. Gas chromatography-mass spectrometry (GC-MS) separates chemical mixtures (using the GC component) and identifies the components at a molecular level (using the MS component).

GC works on the principle that a mixture can be separated into its individual components on the basis of their partition coefficients between the mobile and stationary phases. A sample for analysis is vapourised and carried through a long capillary column (metres) holding the stationary phase by an inert gas such as helium. The constituent molecules of the sample partition into the stationary phase according to their partition coefficients between the stationary and

mobile phases. When in the stationary phase they do not travel through the column and so substances with a high partition coefficient move more slowly through the column than those with a low partition coefficient. Temperature plays an important role in influencing the speed of analyte separation on GC which is applicable for compound that are stable and can volatilised at temperatures up to 350°C.

As the separated substances emerge from the column opening, they flow into the mass spectrometer or into a detector such as the flame ionisation detector (FID) and electron capture detector (ECD). Mass spectrometry identifies compounds using the characteristic distribution pattern of the analyte molecule and its fragment ions, usually displayed as the mass spectrum.

GC-MS is normally used to separate volatile organic compounds including drugs and pesticides. In forensic toxicology GC-MS is a sensitive and specific method for drug determination in biological fluids such as blood, urine and hair due to generation of reproducible and repeatable mass spectra which can be matched with spectra in the available libraries.

Even though GC-MS has been considered as a gold standard technique in forensic toxicology, there are a few limitations reported such as being unsuitable for the determination of non-volatile or thermally labile substances and often the analytes of interest need to be derivatised using toxic chemical derivatising agents.

The rapid growth of ketamine misuse worldwide has led to the development of various methods of biological sample analysis, mostly using GC-MS [98, 101,102, 103, 104,105,106] . A comparison of the methods is shown in Table 3-2.

Table 3-2 Comparison of various GC-MS methods for determination of ketamine and its metabolites

References	98	101	102	103	104	105	106
Matrix	Monkey urine	Human hair	Human urine	Human urine	Human urine	Human urine	Human urine
Extraction/ preparation	Enzymatic Hydrolysis SPE (HCX Isolute®)	LLE	Acid hydrolysis, LLE	LLE	SPE, Drug-Clean ™	LLE	SPE, Oasis HLB
Linearity (ng/mL)	0-2000	0.05-100 (K) 0.005-10(NK)	0-500	100-5000	50-1000	Not mentioned	30-1000
LOD (ng/mL)	5 pg/mL (K) 5 pg/mL (NK)	0.02 for K & NK	1 (K) 5(NK)	3-75 for K & NK	25 for K & NK	3-10	1 for K,NK, DHNK
LOQ (ng/mL)	20 (K) 50(NK) pg/mL	0.05 for K & NK	5(K) 10(NK)	9-100 for K & NK	50 for K & NK	5-50	15(K) 10(NK) 20 (DHNK)
Precision (%) Intra-day Inter-day	Not mentioned	< 8.1 for both intra- & inter-day precision	<2.1 <5.1	< 12.3 for real samples	Inter- and intra- < 15	Not mentioned	RSD < 8.1
Screening method	Neogen® ELISA	None	None	None	None	None	Neogen® ELISA
Identification mode	Chemical Ionization (CI) mode	SIM Mode	SIM Mode	Positive ion chemical ionization (PCI)	Positive ion chemical ionization (PCI	Full scan SIM mode	SIM mode
Accuracy (%)	Not mentioned	80 to 98(K) 82.5 to 117.5 (NK)	93.7-102.5	Not mentioned	78-116	Not mentioned	90-104
Derivatization	None	None	None	Penta-flouro-benzoyl chloride (PFBC)	None	Comparison of 10 agents	None

3.2.2 High Performance Liquid Chromatography (HPLC)

HPLC is an analytical technique that complements other chromatographic procedures such as GC for the quantification of drugs and their metabolites in biological fluids. HPLC is preferred for polar, less volatile and thermally labile compounds. Generally, HPLC is a technique for the separation of mixtures by using a variety of chemical interactions between the analyte and the column.

A small volume of dissolved sample is introduced into the stream of mobile phase passing through the column by means of a valve injector holding a constant or variable volume sample loop that is loaded with a syringe manually or from an auto-sampler. The sample components are retained by specific chemical or physical interactions with the stationary phase as they traverse the

length of the column. The degree of retention depends on the nature of the analyte and stationary phase and on the mobile phase composition.

In HPLC analysis, the mobile phase is either a single solvent or a solvent mixture which has been degassed and filtered prior to delivery to the column under pressure supplied by a constant flow pump. The function of the pressure is to increase the linear velocity which gives the components less time to diffuse within the column and improves the resolution of the chromatogram. HPLC columns are shorter (centimetres) than GC columns and made of stainless steel tubing packed with chemically modified silica, unmodified silica, polymeric resins or gels. The time at which a specific analyte emerges from the end of the column is called the retention time and is an identifying characteristic of a given analyte.

The analysis can involve either isocratic elution, if the mobile phase composition is the same throughout the run time, or gradient elution, if the mobile phase composition is changed according to a predetermined programme. Apart from the column, the pump is the heart of the equipment and supplies pressure for a consistent and reproducible flow rate. The choice of mobile phase, additives and gradient depends on the nature of the stationary phase and the analyte(s) of interest. Variable-wavelength and diode array ultra-violet (UV) detection are commonly used in forensic toxicology. Other types of detector encountered include fluorescence and refractive index detectors.

A number of methods using HPLC have been developed for the quantification of ketamine and its metabolites in biological fluids, mostly in human plasma, to measure the analyte, the metabolites or their enantiomers after medicinal use in patients such as anaesthetic or analgesic. These methods showed excellent selectivity, reproducibility, speed and simplicity. Another distinct advantage is that only a small sample volume and less pre-treatment of the samples are required for the analysis [107 ,108, 109,110,46,111]. The characteristics of each method are shown in Table 3-3.

Table 3-3 : HPLC method for ketamine and metabolites determination

References	107	108	109	110	46	111
Matrix	Human plasma	Human plasma	Human plasma	Human plasma	Equine serum	Human plasma
Extraction/ preparation	SPE, Oasis HLB	LLE	LLE	SPE using Sep-Pak light C18 cartridge	Micro separation with a 10 000 mass cut-off filter	LLE (cyclohexane)
HPLC Column	Monolithic silica	Reversed-phase Ultremax 5 µm CN	Chiralcel OD	Chiral AGP	Phenyl sphrisorb	Chiral AGP (Assay I) Nucleosil RP (Assay II)
Mobile-phase	Monobasic sodium phosphate: Acetonitrile (75:25,v/v)	Methanol: acetonitrile: orthophosphoric acid: 0.01 M sodium dihydrogen-phosphate (200:80:2:718, v/v/v/v)	n-hexane: 2-propanol (98:2,v/v)	10 mM potassium dihydrogen phosphate buffer pH 7: methanol (84:16,v/v)	acetonitrile: ortho-phosporic acid: water (20:2:78,v/v/v)	Assay I 2-propanol: 0.02 M Phosphate buffer, pH 7 (2.5:97.5,v/v) Assay II Acetonitrile: 0.03 M Phosphate buffer, pH 7.5 (30:70,v/v)
Linearity (ng/mL)	25-2000(K) 25-1500(NK) 15-750(DHNK)	10-400 (K)	10-500	10-350	50-5000	50-5000 (K) 50-500(NK)
LOD (ng/mL)	12.5 (K & NK) 7.5 (DHNK)	1 (K)	5 =K enantiomers 10=NK enantiomers	2 =K & NK enantiomers	K=15 (UV) K=5 (PDA)	40
LOQ (ng/mL)	25 (K & NK) 15 (DHNK)	10 (K)	Not mentioned	Not mentioned	Not mentioned	Not mentioned
Precision (%) Intra-day (ID) Inter-day (IDa)	ID =1.3 -7.2 IDa =1-5-8.7	ID =<11 IDa = <13.3	ID = 2.9 9.8 IDa =3.4 - 10.7	IDa =4.8 (S-K) IDa =6.0 (R-K) IDa =8.5 (S-NK) IDa =4.1(R-NK)	Not mentioned	IDa = < 15 except for 43.3
Identification mode	UV at 220 nm	UV at 215 nm	UV at 215 nm	UV at 220 nm	UV at 215 nm (screening) Photo-diode array (confirm) at 200-320 nm)	UV at 215 nm
Recoveries (%)	95.3 (K) 96.9(NK) 103.9(DHNK)	79.9 (K)	86-1 to 91.3 for K and NK enantiomers	81 (K enantiomers) 87 (NK enantiomers)	106.3 (K)	89.3 - 101.4 (Assay I) 74.8-103.9 (Assay II) for K and NK enantiomers
Significance	Application of monolithic silica column for ketamine, norketamine and dehydro-norketamine detection	To measure ketamine & bupivacaine simultaneously at low analgesic doses	Method to determine ketamine enantiomers and norketamine in human plasma	Method to determine sub-anaesthetic concentration of ketamine and norketamine enantiomers in human plasma	Method to determine Ketamine and dehydro-norketamine in equine serum	Comparison of 2 methods emphasized on reverse-phased column to determine concentration of ketamine and norketamine enantiomers in human plasma

However, HPLC also has some limitation in the identification of some drugs such as overlapping of analyte peaks, poor absorption of UV light of some analytes in

the analysis using the UV and diode array detector (DAD); and also absorbance of some analytes are affected by mobile phase composition such as pH and solvent strength.

3.2.3 Liquid Chromatography Mass Spectrometry (LC-MS)

Remarkable developments have extended the applicability of HPLC to robust, efficient and routinely used LC-MS instruments. The technology solves the problem of the inherent lack of sensitivity of the ultra violet detector for a number of drugs and metabolites by providing a more universal detector in mass spectrometry, for more sensitive detection and quantification.

LC-MS methods can be used to analyse a significantly larger number of organic compounds than GC-MS, including those which are polar, less volatile and thermally labile. The most common application of LC-MS is to the identification and quantification of specific compounds in mixtures [112]. Currently, it is increasingly used for screening purposes in many laboratories as an alternative to immunoassay [113,114,115]. LC-MS eliminates the need for time-consuming derivatisation step often required in GC-MS.

LC-MS is an analytical technique that combines the physical separation capabilities of liquid chromatography (LC) which provides the chromatographic separation of analytes from the matrices with the mass analysis capabilities of mass spectrometry (MS) which is used to obtain mass to charge ratios (m/z) or resolution of the analytes of interest from the matrices (detection of analytes). LC is useful for removing the interferences from the sample that would impact on the ionization and mass spectrometric detection offers high sensitivity and a high degree of selectivity compared to conventional HPLC detectors.

The vital part of this combined technique is the interface between the chromatograph and the mass spectrometer which involves various ionisation techniques such as Atmospheric Pressure Chemical Ionisation (APCI), Electrospray Ionization (ESI), Thermospray, Fast Atom Bombardment (FAB) and Nanospray Ionization (NSI). Most LC-MS applications today use atmospheric-pressure ionization (API) techniques which are of two types - ESI and APCI as the interface element to introduce the liquid sample into the ion source. These two interface techniques are robust and widely used in forensic toxicology and are

described frequently in the literature [116,117,118,10,31]. APCI and ESI convert sample molecules to ions at atmospheric pressure and then transfer the ions to the high vacuum environment of the mass analyzer.

ESI is considered a soft ionization technique which produces few ions, usually consisting of a protonated molecular ion plus some adduct ions involving constituents of the mobile phase. This technique transforms ions in a liquid into ions in the gas phase and was used in this study. The theory will be explained in Chapter 5 [119]. The major disadvantage of the ESI interface is in fact the lack of product ions, as often only a pseudomolecular ion is produced. This causes problems in satisfying accepted criteria for the identification of analytes and has resulted in the advent of tandem mass spectrometry (MS/MS), which allows the molecular ion to be fragmented in secondary reaction processes. This will also be covered in more detail in Chapter 5.

In the APCI interface, vaporised solvents act as the reagent gas. The sample and eluent are nebulised in a hot chamber where reagent ions are formed by the interaction of solvent molecules with a corona discharge. Reagent ions interact with sample molecules via protonation or change transfer to produce sample ions. Sample ions pass through skimmers into a mass analyzer. To vaporize the sample, the chamber is heated to 200°C – 500°C. Higher temperatures will decompose the sample. ESI is softer ionisation technique therefore leads to less thermal degradation than APCI.

As shown in Figure 3-1, the ESI interface is preferred to APCI due to its broader range of applications [120]. ESI-MS is sensitive for the detection of high molecular weight compounds such as proteins and peptides. The interface is also suitable for highly polar compounds or thermolabile molecules such as glucuronide conjugates and quaternary amines [121].

The interest in LC-MS applications also led to the development and improvement of mass analysers including the quadrupole mass analyser, quadrupole ion trap mass analyser and time-of-flight mass analyser. A quadrupole ion trap mass analyser was used in this study.

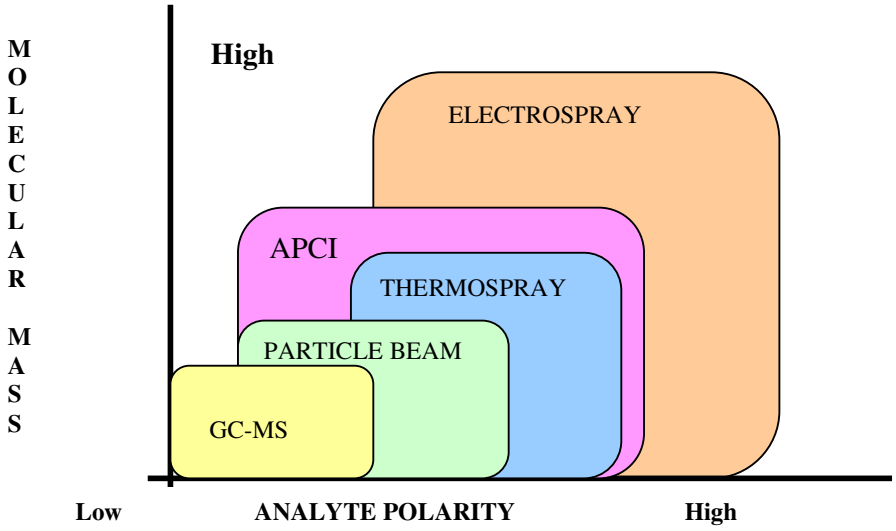


Figure 3-1 Molecular weight and polarity versus the choice of interface

There are a few publications on the determination of ketamine, its metabolites and their enantiomers by LC-MS in various samples such as urine and human plasma which showed very good sensitivity, specificity and recovery with a short running time. Cheng et al. [116] developed a method for ketamine determination in urine which was rapid (2.5 min analysis time) and had a good recovery (89 %) and good precision (11 % RSD) at 20 ng/ml. Urine samples were extracted by automated SPE using a Gilson XL4 with Alltech cartridges, which obtained a 5 ng/ml detection limit and could analyse up to 200 samples per day. The samples were analysed with LC coupled to a Finnigan LCQDUO ion trap mass spectrometer with an electrospray ionization source.

Lua et al. [122] developed a rapid screening method with a 1.3 min run time per sample which involved LLE in 0.2 ml hexane and analysis by LC-ESI-MS operated in full scan mode. The detection limits were 3 ng/ml for ketamine and 15 ng/ml for norketamine with within-run precision at 3 levels of 4.0 % to 14.7 %. The sensitivity and specificity were 97.1 % and 85.7 % when coupled with a GC-MS confirmation method for detection of 168 urine samples from disco-dancing club participants.

A sensitive method of determination of ketamine and norketamine in human plasma by micro-liquid chromatography mass-spectrometry has been developed

by Legrand et al.as part of a clinical trial in a paediatric population [123]. The analytes were extracted using solid phase extraction on Oasis MCX® cartridges and separation of analytes was performed on a C-18 micro-column using a mobile phase of formic acid 0.1 % and acetonitrile (90:10, v/v) with a run time of 10 min. The method linear range was 50 to 500 ng/ml and recoveries were from 98.1% to 101.7 %. Mass spectral data were obtained using selected ion monitoring (SIM) mode and limits of quantification were 4 ng/ml for both ketamine and norketamine.

A group of researchers in the USA [124] developed a method for determination of ketamine and norketamine enantiomers in human plasma for a study of ketamine in pain management. Extraction was by solid phase extraction (Oasis HLB®) prior to direct injection to a LC-MS system and the method could process up to 60 patient samples per day. The enantioseparation was performed on a chiral AGP column using an isocratic mobile phase of 2-propanol-ammonium acetate buffer (10 mM, pH 7.6) with a composition of (6:94,v.v) and a flow rate of 0.5 ml/min. Chromatographic identification and quantification were carried out using SIM mode. The method had a linearity of 1 - 125 ng/ml and an analysis time of 20 min, with intra- and inter-day precision < 8.0 %. The authors claimed the sensitivity was better than in previously reported methods for determination of ketamine and norketamine enantiomers.

3.3 Techniques for Sample Preparation

Biological fluids such as blood, plasma or urine are referred to as complex matrices and normally contain a number of endogenous components. These matrix components may interfere with and may adversely affect the subsequent separation and identification of analyte(s) of interest if they are not removed. Therefore, two-thirds of the total analysis time in chromatographic methods is typically due to the sample preparation steps and these are often the major source of error in the overall analytical process.

Sample preparation impacts on nearly all the later assay steps and is hence critical for unequivocal identification, confirmation and quantification of analytes. It includes both the isolation and/or pre-concentration of compounds of interest from various matrices as well as making the analytes more suitable for separation and detection. Extraction procedures are critical preparation

processes in analytical methods and identification and separation of compounds cannot be achieved without proper treatment of the samples.

A few types of extraction method are used routinely in analytical toxicology such as Liquid-liquid Extraction (LLE), Solid Phase Extraction (SPE), Solid-Phase Micro-Extraction (SPME), and Molecularly Imprinted Solid Phase Extraction (MISPE). Currently, the most convenient method for extraction of drugs in biological fluids is SPE due to its simplicity and versatility which leaves LLE, which is labor intensive and solvent consuming, far behind in analytical methodologies. MISPE is currently a growing field in clean up techniques for the analysis of biological samples. SPE and MISPE were investigated and evaluated in this thesis.

3.3.1 Solid Phase Extraction (SPE)

3.3.1.1 Introduction to SPE

SPE started in the early 1970s but during the 1980s and 1990s development of SPE methods and their use in analytical chemistry greatly increased [125]. Nowadays, SPE is well established and widely used in many different fields of analytical chemistry including forensic toxicology. SPE is a very common type of sample extraction and clean up technique for biological fluids in analytical chemistry and a well-accepted sample preparation technique in the analytical chemistry community [125].

It is a rapid sample preparation method used to selectively extract, concentrate and purify target analytes prior to further analysis by chromatographic techniques. It is a form of solid absorption chromatography ranging from simple hydrophobic or hydrophilic partition chromatography to ion-exchange or affinity chromatography. SPE is a very simple technique to use, employing inexpensive, disposable extraction columns that are available in a multitude of column sizes and sorbents. SPE cartridges are available in tube, cartridge and disk formats.

3.3.1.2 Principles of SPE

Sample preparation with SPE consists of four basic steps: conditioning, sample loading, washing and elution. The aim is to retain the analyte in the sorbent

bed, wash away interferences and finally elute the analytes as a clean extract in a small volume prior to analysis by suitable method such as LC-MS.

In principle, a sample is loaded on an SPE column. The sample matrix and interferences pass through the column while analytes are retained on the sorbent material in the column. Interferences can then be selectively removed from the column through the correct choice of wash solvents. Finally, the analytes may be selectively recovered from the column by an elution solvent, resulting in a highly purified extract. Alternatively, an extraction column may be selected which retains the interferences in the sample, but allows the analytes to pass through unretained.

Most SPE columns contain sorbents with an average particle size of 45-65 μm base material. Most organic solvents will flow through the columns under gravity, but for aqueous and other viscous samples and solvents, liquids must be passed through the columns using vacuum applied to the column outlet, pressure applied to the column inlet, or centrifugation. A vacuum manifold is the most commonly employed method.

These techniques can be used either off-line or on-line. In the on-line mode, the sorbent is packed into a pre-column which is located in a six-port switching valve connected to an LC system. In the load position, SPE is performed and, in the inject position; the pre-column is connected to the analytical column. This allows simultaneous elution and transfer of the retained analytes by the mobile phase of the LC separation. The on-line coupling of SPE with an LC system provides higher enrichment factors compared to the off-line system, when the eluent is evaporated and reconstituted before being injected into the analytical column.

3.3.1.3 SPE Sorbents

SPE is performed using either silica-based or polymer-based sorbents. The nature of the base material and the functional groups both affect the way that the sorbents are used. Silica-based sorbents have a number of advantages including rigid support particles that do not shrink or swell and the availability of a larger selection of phases. Polymer-based sorbents have different advantages including

lack of the need for acidic/basic elution modifiers, no pH limitations (stable from pH 1-14) and high capacity.

There are a number of phases to suit different SPE applications [126]. Polar (normal phase) sorbents extract polar to moderately polar compounds from non-polar matrices using organic eluents such as acetone, chlorinated solvents and hexane. Aminopropyl (NH_2), cyanopropyl (CN), diol, silica (Si), florisil and alumina are polar phases for normal phase interaction. Retention of an analyte under normal phase conditions is primarily due to interactions between the polar functional groups of the analyte and polar groups on the sorbent surface. These include hydrogen bonding, Δ-Δ interactions, dipole-dipole interactions and dipole-induced dipole interactions, among others. A compound adsorbed by these mechanisms is eluted by passing a solvent that disrupts the binding mechanism, usually a solvent that has polarity more than the analyte's original matrix.

Reversed phase SPE sorbents extract moderately polar to non-polar analytes from aqueous matrices using a non-polar stationary phase, for example the alkyl- or aryl-bonded silicas. Octadecyl (C_{18}), octyl (C_8), cyclohexyl (CH), phenyl (PH), butyl (C_4) ethyl (C_2) and methyl (C_1) are non-polar phases for reversed phase interaction. Retention of organic analytes from polar solutions (e.g. water) on these sorbents depends on lipophilic interactions (van der Waals forces, or dispersion forces) between the carbon skeleton in the analyte and the functional groups on the silica surface. To elute an adsorbed compound from a reversed phase SPE cartridge, a non-polar solvent is used to disrupt the forces that bind the compound to the packing.

A few studies have successfully used this type of sorbent to extract ketamine and metabolites from biological sample matrices such as CleanScreen® (C_8) [104], Oasis HLB® which contained an equal hydrophilic-lipophilic reversed phase sorbent [106, 107,124], Sep-Pak C_{18}® [110] and Alltech C_{18}® [116].

Ion exchange sorbents separate analytes based on electrostatic interactions between the analyte of interest and the positively charged groups on the stationary phase. Both the stationary phase and sample must be at a pH where both are ionised.

Anion exchange sorbents are derivatized with positively charged functional groups, such as amines, that interact and retain negatively charged anions, such as acids, while cation exchange sorbents are derivatized with functional groups, such as acids, that interact and retain positively charged cations, such as amines [127] . Benzenesulfonic acids (SCX), quaternary amine (SAX), aminopropyl (NH_2) and primary/secondary amine (PSA) are examples of ion exchangers.

Mixed mode sorbents contain both hydrophobic and ionic functional groups which enable the sorbent to provide two types of retention mechanisms. This type of sorbent is widely used in forensic toxicology because of its high selectivity and ability to provide clean chromatograms. Sorbents functionalised as mixed-mode cationic exchangers which have high selectivity for basic compounds have been used in few studies to obtain high recoveries of ketamine and its metabolites from complex sample matrices such as Isolute HCX® [40] and Oasis MCX® [57].

Polymer based SPE involves macroporous polymeric media, offering a higher loading capacity compared to conventional functionalized silica SPE media. They are compatible with all polar and non-polar, protic and aprotic solvents and can be used over a wide pH range, pH 1-14. One example is Strata™ X which has the advantage of having no residual surface silanol groups, unlike the substituted silica sorbents. The structure of Strata™ X is shown in Figure 3-2.

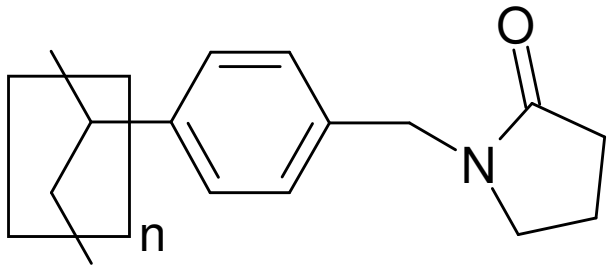


Figure 3-2 Structure of Strata™ X

SPE techniques have proved to give a better selectivity and specificity for the target compound(s) and to result in higher recoveries and reproducibility due to the wide range of sorbents available in the market. SPE also has higher concentration factors and can be automated, and this enables an analyst to process multiple samples rapidly. SPE only requires a small volume for

extraction compared to LLE. In conclusion, SPE not only offers more advantages over LLE in terms of performance but also in economic and environmental benefits.

3.3.2 Molecularly Imprinted Solid Phase Extraction (MISPE)

A Molecularly Imprinted Polymer (MIP) is a synthetic polymer bearing on its surface the molecular imprint of a specific target molecule (the template). The main advantage of molecular imprinting is the possibility of preparing a selective sorbent pre-determined for a particular substance or a group of structurally related substances. The polymer has a permanent memory of the template and is capable of selectively rebinding the template or structurally similar molecules such as the metabolites [128].

The application of MIPs as sorbents in SPE has been found very useful for selective extraction for the reason that MIPs offer higher selectivity than conventional SPE. MIPs also reduce the influence of the matrix on the resulting chromatogram and high sample enrichment factors are achieved. The work on MISPE was started by Sellergen in 1994 who successfully extracted pentamidine, a drug use to treat pneumonia for AIDs patients, from human urine using a MIP material as the SPE sorbent [129].

From then, several applications of MISPE have been successfully employed in the determination of environmental and pharmaceutical samples such as nicotine in chewing gum[130] and atrazine in beef liver [131] , in which MISPE was used to clean-up the extract and concentrate the analyte for further analysis. This removed the interfering components in the sample and subsequently improved the accuracy of HPLC analysis and lowered the detection limit.

MISPE has not been used in forensic toxicology until recently. Ariffin et al [132] developed a method for MISPE prior to analysis using LC-MS for benzodiazepine detection and found it to be simpler and cleaner compared to conventional SPE. The work of Ariffin et al. opened the door to the application of MISPE in forensic toxicology. The work in this thesis applied MISPE methods to ketamine extraction and compared them to conventional SPE techniques.

In this study, an MIP prepared with (±)-ketamine as a template was used in a wholly novel sample preparation method for the analysis of ketamine and norketamine in hair based on molecularly imprinted solid-phase extraction (MISPE).

SupelMIP® is the first MIP-based SPE sorbent which offers tailor-made selectivity for the extraction of trace analytes in complex matrixes and the first to be commercialised in year 2008 by Supelco. Part of the present work investigated the potential of Amphetamines SupelMIP® for the determination of amphetamines in post-mortem blood.

4 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR SCREENING OF KETAMINE AND ITS METABOLITES

4.1 Introduction

ELISA is the acronym for "enzyme-linked immunosorbent assay." This is a rapid immunochemical test that combines the reaction of an enzyme, a protein that catalyzes a biochemical reaction, with an antibody/antigen reaction. The test also requires an appropriate enzyme label and a matching substrate suitable for the detection system being used.

Generally, ELISA tests show the same high sensitivity and specificity as other immunoassay tests such as radioimmunoassay (RIA). For example, there are established ELISA tests for proteins such as α-fetoprotein and hCG, with limits of detection as low as 1 ng/ml and with good intra-assay precision, with coefficients of variation in the range 8-10 %, which produce performance as good as the corresponding RIA tests [96]. ELISA kits are also safer and have longer usable time periods compared to RIA. The radioisotope commonly used in RIA is iodine-125 [96], which has a short half-life and as a result, a short shelf life, usually a maximum of six months.

An ELISA test may be interpreted in a qualitative or semi-quantitative format. Qualitative results provide simple positive or negative results for a sample. The cut-off is a value serving as an administrative break point for labeling a sample as positive or negative and determined by the manufacturer or the analyst. This cut-off value is at least equal to but usually higher than the limit of detection otherwise a high number of false positive results may be obtained. The cut-off value selected should result in a method sensitivity and specificity of better than 90 % [133].

In semi-quantitative ELISA, a serial dilution of the target analyte is prepared and the optical density or fluorescence emission of the end product of the substrate-enzyme reaction is interpolated into a standard curve. The test is called semi-quantitative since the assay result represents the summative contribution from

all drugs from a particular drug class in a sample that can compete for the antibody binding sites and does not generally represent a single target analyte.

ELISA has become popular within the forensic toxicology community because it is relatively easy to perform, has good potential for automation, needs only a small sample size, does not require radioisotopes and can be used with blood, urine and other biological samples such as hair and saliva [134] . Currently, it is the only type of commercially available immunoassay test for the screening of ketamine and its metabolites. The purpose of this study was to evaluate and validate the suitability of the commercially-available Neogen® kit for the screening of samples for ketamine in urine before being confirmed by a quantitative method, liquid chromatography tandem mass spectrometry (LC-MS/MS).

4.2 Principles of ELISA

ELISA is one type of immunoassay test which involves a binding reaction between an antibody and its cognate antigen (the analyte). The kinetics of an antibody-antigen reaction in an immunoassay test can be described by the *Law of Mass Action* as shown in Equation 4-1 and Equation 4-2.

Equation 4.1

$$[Ag] + [Ab] \underset{k_d}{\overset{k_a}{\rightleftharpoons}} [Ag\text{-}Ab]$$

Equation 4.2

$$K_{eq} = \frac{k_a}{k_d} = \frac{[Ag\text{–}Ab]}{[Ag][Ab]}$$

where,

[Ag] = antigen concentration,
[Ab] = antibody concentration,
[Ag-Ab] = antigen-antibody complex,
k_a = association rate constant,
k_d = dissociation rate constant.
K_{eq} = equilibrium constant or affinity constant

K_{eq}, represents the ratio of bound to unbound analyte and antibody. This ratio can be translated into a signal that can be measured spectrometrically at a selected wavelength.

There are several different types of ELISA test, including either a competitive or sandwich type. In competitive ELISA, an affinity-purified capture antibody such as anti-IgG is pre-coated onto a microplate. A limited concentration of primary antibody and enzyme-linked analyte along with the sample are added simultaneously. Sample analyte and enzyme-linked analyte compete for the limited number of binding sites on the primary antibody, which is bound by the immobilized anti-IgG. Substrate is added and hydrolyzed by the enzyme, thereby producing a colour product that can be measured. The amount of labelled analyte bound is inversely proportional to the amount of unlabeled analyte present in the sample (signal decreases as analyte concentration increases, Figure 4-1) [135] . This type of assay was used in the present study.

The sandwich technique is so called because the antigen being assayed is held between two different antibodies. An antibody for the target analyte is coated to a solid support such as microplate wells, then a sample containing antigen is added. The mixture is incubated to allow the Ag-Ab reaction to take place before the unbound components are washed away. Thereafter, a known amount of antibody for the target analyte which has been labelled with enzyme is allowed to react with the bound antigen to form an Ab-Ag-Ab complex. Any excess unbound enzyme-linked antibody is washed away after the reaction. The substrate is then added and the reaction between the substrate and the enzyme produces a colour change. The amount of visual colour change is directly proportional to the amount of specific enzyme-conjugate bound antibody, and consequently to the concentration of antigen present in the specimen tested [136].

The Neogen® direct competitive ELISA ketamine assay operates on the basis of competition between the drug or its metabolite in the sample and the drug-enzyme conjugate for a limited number of specific binding sites on a surface, often a microplate pre-coated with antibodies specific to the target analyte.

The simple assay procedure includes the addition of samples or controls to the well of an antibody-coated microplate. The antibody is fixed to the surface to render it immobile. The enzyme-ketamine conjugate is added to the mixture and this is followed by an incubation time of selected duration. During incubation, ketamine in the sample and ketamine-enzyme conjugate compete for binding

sites on the antibody-coated microplate. After the incubation, the microplate is washed, so that unbound ketamine is removed and only ketamine-antibody complexes remain attached to the well. Subsequently, a substrate for the bound enzyme is added followed by another incubation time. The enzyme converts the substrate to elicit a chromogenic signal. After the incubation, a stop solution is added to end the reaction and the test is read visually or with a microplate reader.

The extent of colour development is inversely proportional to the amount of drug in the sample or control. The absence of the drug in the sample will result in a more intense colour, whereas the presence of the drug will result in decreased or no colour development [137] . A schematic presentation of the test principles of the Neogen ketamine ELISA used in this study is depicted in Figure 4-1.

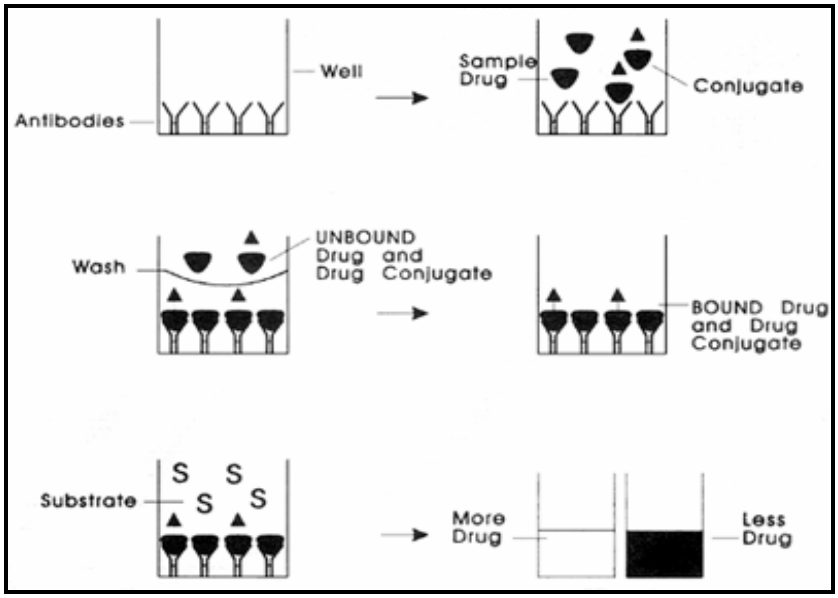


Figure 4-1 The test principles of Neogen ketamine ELISA (copyright Neogen Corporation®)

4.3 Experimental Section

4.3.1 Chemicals and reagents

Ketamine ELISA kits (product number: 109419) were purchased from Neogen® Corporation (Lexington, USA). The kits contained 96 antibody-coated microplate

wells, wash buffer (phosphate buffered saline solution, pH 7, containing a surfactant), ketamine-enzyme conjugate labelled with horseradish peroxidase (HRP), 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution with hydrogen peroxide (H_2O_2), a red stop solution containing 0.5 M H_2SO_4, as well as negative (0 ng/mL) and positive (2000 ng/mL) controls prepared in synthetic urine. Phosphate buffer saline (PBS) pH 7 was purchased from Immunolysis® Corporation (Pomona, CA, USA) which contained bovine serum albumin and non-azide preservatives. IM HCl was used as the stop solution instead of 0.5 M H_2SO_4 provided by the manufacturer to fit the Tecan Reader available in the laboratory.

Ketamine, norketamine and drugs standards used to test the cross-reactivity of the ELISA kit, namely amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylene-dioxymethylamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), cocaine, benzoylecgonine, diazepam, nordiazepam, morphine, methadone, 6-monoacetylmorphine (6-MAM) and phencyclidine (PCP) were obtained from Promochem (Teddington, UK). Tiletamine.HCl powder was purchased from Sigma-Aldrich (Dorset, UK).

4.3.2 Equipment

A miniprep 75 automatic pipettor purchased from Tecan (San Jose, CA) was used to dilute the samples, controls and calibrators and to pipette them into microplate wells. A Sunrise Remote EIA Reader from Tecan was used to read the microplates at a wavelength of 450 nm. Disposable borosilicate glass culture tubes (75 x 12 mm) from VWR International, Poole, UK were used for samples and dilutions. Washing was done manually.

4.3.3 Preparation of solutions [138]

4.3.3.1 Enzyme conjugate

The enzyme conjugate supplied with the Neogen® kit was diluted by a factor of 180 with the EIA buffer (PBS: phosphate buffered saline. pH 7). The volume needed was calculated. For example, two eight well strips needed 2880 µl (2.8 ml) and 25 µl enzyme conjugate stock was added to 4475 µl of EIA buffer. The

solution was mixed by inversion. The enzyme conjugate was freshly prepared before use.

4.3.3.2 Wash buffer

The concentrated wash buffer was diluted 10 fold with deionized water. For example, 20 ml of concentrated wash buffer was mixed thoroughly with 180 ml deionized water. Diluted wash buffer is stable in room temperature for 5 days or 7 days at 4°C.

4.4 Method Development

In ELISA, several requirements are associated with the enzyme-substrate reaction, such as timing and development conditions that need to be optimised to get a precise, accurate and reproducible assay. There were a few conditions applied in this study to get the lowest percentage of colour activity (B/B_0) where B is the absorbance reading of the bound calibrator and B_0 is the absorbance value of the blank calibrator.

The three most important factors evaluated in the study were the volume of sample, volume of enzyme-conjugate and the pre-incubation time. For the 12 conditions, urine spiked with ketamine at a concentration of 25ng/ml was used with n=5. During method development, specimen volume (20 or 40 µl), enzyme conjugate (90 or 180 µl) and pre-incubation time (0, 30 or 60 min) were evaluated. The experimental conditions used and the results are tabulated in Table 4-1.

In this study, the parameters which proved to give the best distribution of absorbance and concentration values and showing the widest difference in absorbance between the blank and the 25 ng/ml were used for the subsequent validation study. The data in Table 4-1 show that that pre-incubation time was not a very significant factor and 0, 30 and 60 min produced quite similar results.

The most important factor was the volume of enzyme conjugate used: the higher volume (180 µl) was better than the lower volume (90 µl) and gave a decrease of approximately 10 % in the B/B_0 value. 40 µl of sample gave slightly better results

than 20 µl but still in the same B/B_0 absorbance range. For forensic toxicology purposes, where there is a need to have lower sample volumes and shorter analysis times, the parameters chosen as a result of this study were 20 µl sample, 180 µl enzyme conjugate solution and no pre-incubation time.

This finding was similar to a study for the optimization of an ELISA method for cocaine in hair carried out by Lachenmeier et al., who tried 20 µl and 50 µl sample volume, 50 and 100 µl enzyme conjugate and 15, 30 and 45 minutes pre-incubation time and found that 20 µl sample, 100 µl enzyme conjugate and no pre-incubation time were the parameters that gave the desired target and which were used in the remainder of the study [139]. The optimized parameters selected in the present work also tallied with the procedures recommended by the manufacturer.

Table 4-1 Experimental conditions for method optimisation

Experimental conditions Urine diluted (1:10)			Average B/B_0 (%)	STD DEV	% RSD *
Volume of sample (µL)	Volume of enzyme conjugate (µL)	Pre-incubation Time (min)			
20*	90	0	84.16	3.09	3.67
		30	84.38	1.37	1.63
		60	83.95	1.63	1.94
	180*	0 *	74.07	3.09	4.17
		30	74.58	1.73	2.33
		60	74.42	0.94	1.26
40	90	0	83.52	3.12	3.74
		30	82.93	1.09	1.31
		60	80.85	1.65	2.04
	180	0	73.94	2.22	3.00
		30	72.14	3.12	4.32
		60	71.52	1.75	2.45

*** Parameter used for validation**

*** % RSD = (SD/ Average B/B_0) x 100**

4.5 Method Validation

Method validation procedures used in this study were based on published papers dealing with the validation of ELISA methods for drugs in the forensic toxicology field [24,140] and also with reference to validation guidelines for a semi-quantitative method [141]. The ELISA kit manufacturers determined a plate sensitivity of 8 ng/mL ketamine in buffer compared to 10 ng/mL ketamine in neat urine (1.25 fold lower sensitivity). Therefore the blank, calibrators and quality control samples were prepared in blank urine for this study in order to take the urine matrix effect into account.

To minimize the effect of incubation time, pipetting was automated using a Tecan miniprep 75 automatic pipettor to get the enzyme conjugate, substrate and stop solution delivered to all of the microplate wells at the same time.

4.5.1 Sample Treatment

I ml of each urine sample was added to separate disposable borosilicate glass culture tubes (75 x 12mm). The samples were vortex mixed then diluted 1:10 with Neogen EIA buffer. 9 parts of EIA buffer (900 µl) were added to 1 part (100 µl) of sample. This procedure was performed to reduce the natural background as well as to bring the cut-off concentration within the assay range. A miniprep 75 automatic pipettor was used to dilute the samples, controls and calibrators and to pipette them into a series of clean disposable borosilicate glass culture tubes. After that, the diluted samples were again vortex mixed.

4.5.2 ELISA Method

The ELISA procedure used in this study was the optimised method with no pre-incubation time; lower sample volume and acceptable B/B_0 (%) values. 20 µl of diluted samples, calibrators or controls were added to the microplate wells, followed by 180 µl of diluted enzyme conjugate.

The plate was then left in the dark at room temperature for an incubation time of 45 minutes for competitive binding between ketamine in the sample and the enzyme labelled conjugate for the antibody binding sites on the wells. After

the incubation, the microplate wells were washed five times with 300 µl diluted wash buffer to remove the unbound sample or residual conjugate that may have been left in the wells. 3,3',5,5'-tetramethylbenzidine (TMB) substrate was then added to the wells and allowed to incubate again for 30 minutes at room temperature in the dark. TMB formed a blue product when allowed to react with peroxidase enzymes such as horseradish peroxidase. The reactions were then stopped with 50 µl of 1M HCl, when the contents of the wells turned yellow and this enabled the chromophore to be detected at 450 nm using the Remote EIA Reader from Tecan. A schematic presentation of procedures used in this study is shown in Figure 4-2.

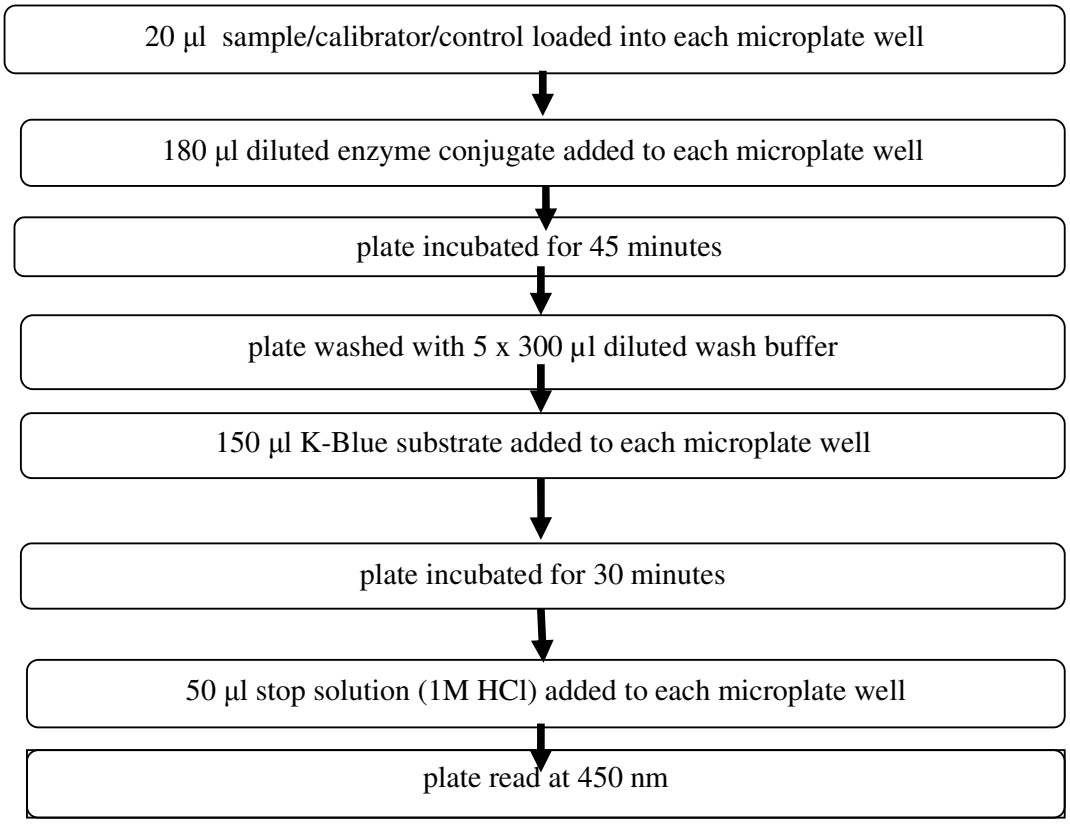


Figure 4-2 Optimised ketamine ELISA assay procedure

4.5.3 Dose-Response Curve

A dose-response curve was generated for 1 ml urine spiked at concentrations of 0, 0.5, 1, 2, 4, 5, 10, 25, 50, 100, 500, 1000, 2000, 4000 and 8000 ng/ml ketamine (Figure 4.3). The test was carried out in duplicate and the average was

plotted. The range of values was selected based on previously published report of ketamine analysis in urine case samples [34,102,105,106,142,143].

The B/B_0 (%) values were calculated where B is the absorbance value of the bound calibrators and B_0 is the absorbance value of the blank calibrator. Both x and y axes are converted into log-scales and the results show that a higher ketamine concentration in the sample resulted in a lower optical density value (B/B_0 %) due to the lower amount of enzyme conjugate bound to the antibody sites.

The graph also indicated that the ketamine assay S-shaped binding curve had a linear portion between 25 ng/mL to 500 ng/mL ketamine, which levelled off after 2000 ng/ml ketamine. The response for the main metabolite, norketamine, was not taken into consideration in this dose response curve due to lower cross-reactivity stated by manufacturer - for 196.4 ng/ml norketamine the cross-reactivity is only 4.6%.

In conclusion, the cut off value 25 ng/ml was selected from one of the lower values within the linear range (25 ng/ml) and any values ≥2000 ng/ml were read as >maximum whereas values below 10 ng/ml would not give very accurate quantitative results.

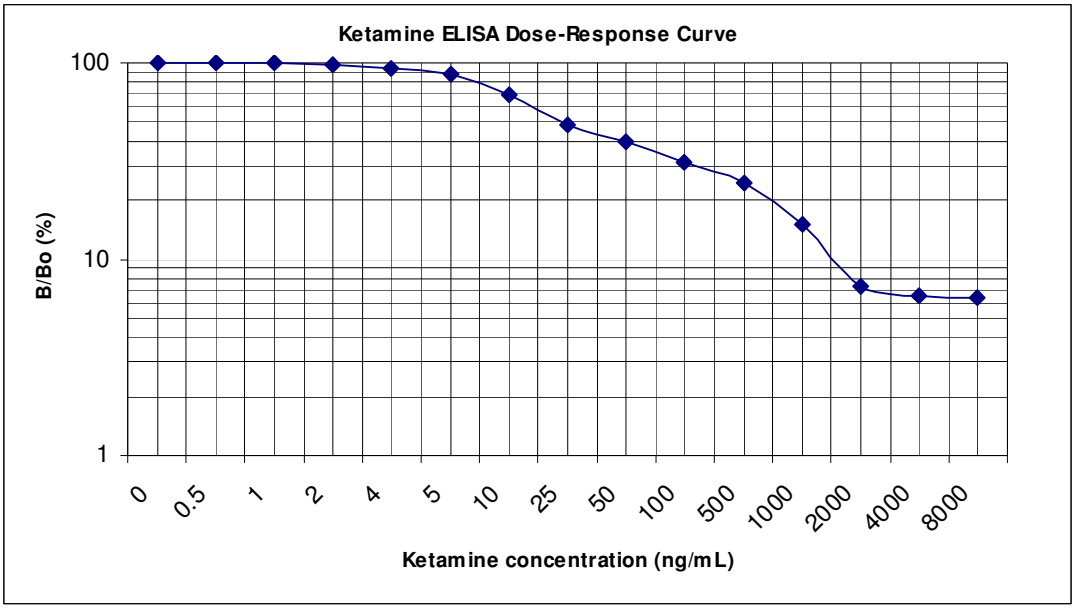


Figure 4-3 Ketamine ELISA dose response curve from 0.5 to 8000 ng/ml.

4.5.4 Intra-day and Inter-day Precision

The intra-day precision of B/B_0 % values was determined by spiking 10 drug free urine samples with 25 ng/ml ketamine. The test was carried out on the same plate and on the same day. For inter-day precision the tests were carried out on five different days with number of samples n=50 and included different batches of reagent kits. The results showed that good precision was obtained from the developed method. The intraday precision for 10 samples spiked with ketamine at 25 ng/ml was 2.5 % RSD and the inter-day precision for 50 samples spiked at the same concentration was 4.8 % RSD. Both intra and inter-day precisions were below 5.0 % and are shown in Table 4-2.

Table 4-2 Intra and inter-day precisions for ketamine ELISA assay in human urine

Intra-day n=10		Inter-day n=50	
Average B/B_0 (%)	RSD (%)	Average B/B_0 (%)	RSD (%)
50.8	2.5	51.8	4.8

4.5.5 Limit of Detection (LOD)

The LOD was calculated using 10 negative samples and Equation 4-3. Ketamine was spiked at concentrations of 0.5, 1, 2, 4, 5, 10, 25 and 50 ng/ml to establish a calibration curve.

Equation 4-3 $LOD = A_0 - 3s$

A_0 is the mean absorbance value and s is the standard deviation of the absorbance values of the negative samples. B/B_0 % of LOD was calculated using Equation 4-3 and converted to concentration of ketamine. This study obtained

an LOD of 5.0 ng/ml for ketamine in human urine using the Neogen® ELISA kit, better than the LOD of 10 ng/ml indicated by the manufacturer in the product insert. However, this LOD was not used as the cut off value in this study to avoid the many false positive results which would probably be obtained with real case samples.

4.5.6 Cross-reactivity

According to the Neogen® ketamine ELISA package insert, norketamine is the only related drug that cross-reacts with ketamine, with a cross reactivity percentage of 4.6 % at a norketamine concentration of 197 ng/mL. However since the ELISA kit is a semi-quantitative test, the assay might have some cross-reactivity to the other metabolite, dehydronorketamine and the conjugates of hydroxylated derivatives of ketamine (glucuronides) which present very high percentage in urine (>80 %).

Ketamine was spiked at concentrations of 0, 2, 4, 5, 10, 20 and 50 ng/ml to establish a calibration curve and norketamine was spiked at 25, 50, 75,150 and 200 ng/ml. The cross-reactivity was calculated relative to the ketamine calibration curve using Microsoft Excel® and the value was found to be 2.1% (Figure 4-4).

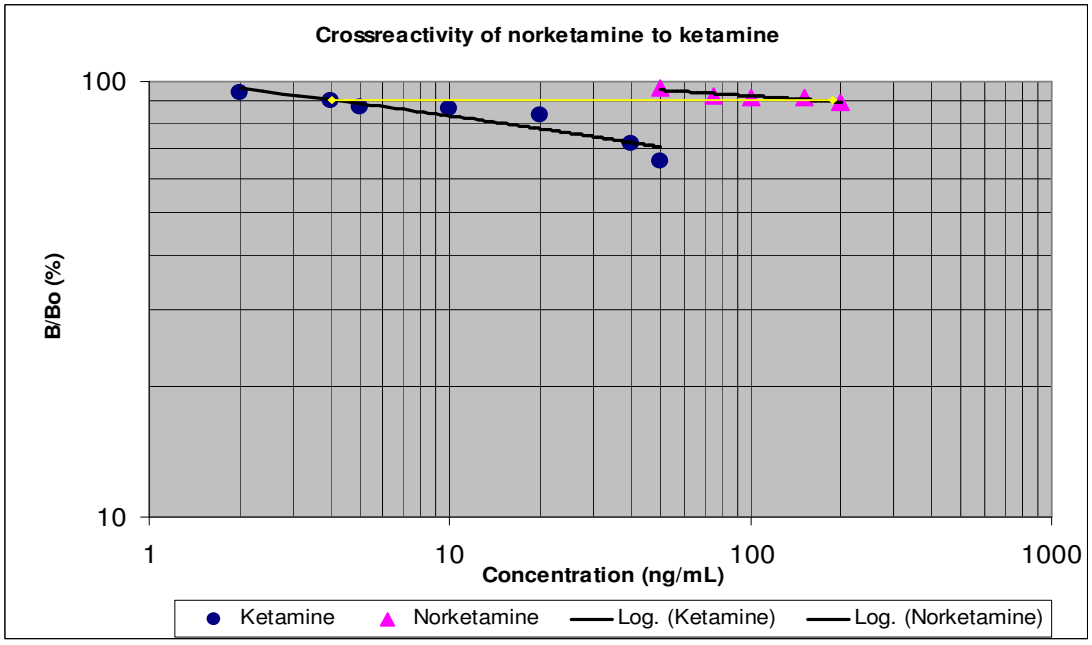


Figure 4-4 The cross-reactivity of norketamine to ketamine in the Neogen ELISA kit

Fourteen commonly abused drugs and an anaesthetic drug (tiletamine) were spiked at a concentration of 10,000 ng/ml to test for cross-reactivity and the results are shown in Table 4-3. Apart from norketamine at very high concentrations, none of the drugs cross-reacted with the ketamine antibody and this would be very useful for screening use.

Table 4-3 Cross-reactivity of norketamine and common drugs tested by the Neogen® ketamine ELISA

Compound	Spiked Concentration (ng/ml)	Cross-reactivity found (%)
Norketamine	200	2.1
AMP	10,000	0
MAMP	10,000	0
MDA	10,000	0
MDMA	10,000	0
MDEA	10,000	0
Cocaine	10,000	0
Benzoylecgonine	10,000	0
Diazepam	10,000	0
Morphine	10,000	0
6-MAM	10,000	0
Methadone	10,000	0
PCP	10,000	0
Tiletamine	10,000	0

4.5.7 Sensitivity and Specificity

The sensitivity and specificity were calculated using Equations 4-4 and 4-5.

Equation 4-5 **Sensitivity = (TP x 100)/ (TP+FN)**

Equation 4-6 **Specificity = (TN x 100)/ (TN+FP)**

A true positive (TP) result produced both positive screening and confirmation results while a false positive result (FP) produced a positive in the screening but negative in the confirmation test. A true negative result (TN) produced a negative result for screening and confirmation test whereas a false negative

result (FN) was negative for screening but positive in the confirmation test. The ideal test is the one with 100% sensitivity and specificity for the target compound but unfortunately most immunoassay tests are sensitive but lack specificity because they can react with compounds of similar structure to the target analyte.

Good sensitivity and specificity of a test can also be translated into a good correlation between screening and confirmation test results. For the ketamine ELISA test, all the screened samples were subsequently confirmed using an LC-MS/MS method which had to be developed and validated in order to calculate the sensitivity and specificity of the ELISA test. The data are presented in Table 5.8 (Chapter 5).

4.5.8 Case Samples

44 samples were analyzed using the ketamine ELISA kit, of which 10 samples screened negative while 34 samples screened positive at a cut-off of 25 ng/ml. They also screened positive at cut-offs of 10 and 5ng/ml. A calibration curve was obtained from urine samples spiked at zero concentration (blank, 0 ng/ml), at the cut off concentration (25 ng/ml), 2 times the cut off concentration (50 ng/ml) and 5 times the cut off concentration (125 ng/ml). Spiked samples with concentrations lower than the cut off (5 and 10 ng/ml) and 10 times higher than the cut off (250 ng/ml) were also analyzed to decide the appropriate cut off for this assay. Negative and positive controls provided by the manufacturer were included to verify the performance of the test.

Negative urine samples were collected from colleagues in Forensic Medicine and Science, University of Glasgow whereas the 34 positive urine samples were obtained from the Narcotics Department, Royal Malaysian Police, collected from people arrested by the police at nightclubs. All positive urine specimens (10 mL volume in each container) were freezed-dried at -54°C by the Drug Laboratory, Pathology Department, Kuala Lumpur General Hospital in Malaysia using a Cole Palmer 1 L bench top freeze-dry system. The lyophilized samples were sent by courier to the forensic toxicology laboratory at the University of Glasgow. Each of the samples was reconstituted with 10 mL deionised water prior to analysis by ELISA. These samples have been previously screened positive using a qualitative

GC-MS method with a ketamine cut-off of 350 ng/mL at the Drug Laboratory, Pathology Department, Malaysia.

The results of the samples tested by ELISA are shown in Table 4-4. All negative and positive screening results by this ELISA method were subsequently confirmed by an LC-MS method which was developed and validated as part of the present study.

Table 4-4 Neogen® Ketamine Microplate ELISA Positive Screened Results.

Sample number	Concentration (ng/ml)	Positive/Negative
1	>125	Positive
2	>125	Positive
3	>125	Positive
4	>125	Positive
5	>125	Positive
6	>125	Positive
7	>125	Positive
8	>125	Positive
9	>125	Positive
10	>125	Positive
11	>125	Positive
12	32	Positive
13	>125	Positive
14	37.4	Positive
15	25.8	Positive
16	29.6	Positive
17	>125	Positive
18	>125	Positive
19	>125	Positive
20	>125	Positive
21	>125	Positive
22	>125	Positive
23	>125	Positive
24	>125	Positive
25	>125	Positive
26	>125	Positive
27	>125	Positive
28	>125	Positive
29	>125	Positive
30	>125	Positive
31	>125	Positive
32	>125	Positive
33	>125	Positive
34	>125	Positive

4.6 Discussion

The increase in ketamine misuse in many countries, as noted earlier in Section 2-1 and Section 2-7, has led to a growing number of clinical and forensic ketamine analyses, using methods which include commercial detection kits available in the market. Pharmacokinetically, ketamine has relatively short distribution and elimination half-lives: the α-elimination phase lasts only few minutes and β-elimination half-life is 2-3 hours. It follows that a rapid, sensitive and reliable method is required to determine ketamine in body fluids [117].

Previously, many analytical methods developed in-house have been performed for the determination of ketamine, such as GC-MS, LC-MS, HPLC-UV and capillary electrophoresis, but there were no commercially available rapid test kits for screening. Only recently, in 2006, the Neogen Corporation produced an ELISA kit for ketamine detection in forensic toxicology.

Immunoassays for drugs of abuse testing need to be rapid and efficient, and to provide high sensitivity and specificity. In this study, the ELISA ketamine test procedures were shown to be rapid and efficient test taking less than 3 hours for analysis of a 96 well microplate, with intra and inter-day precisions of less than 5.0 % RSD.

The optimised test protocol required a minimal sample volume of diluted urine (20 µl) with no pre-incubation time included and could be automated. The manufacturer should has to provide a bigger volume of concentrated wash solution to automate the steps for better performance of the test.

In this study, a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB), was used and the colour change could be read on a spectrophotometer at a very broad dynamic range (a wavelength of 370 or 655 nm). However in this study, the test was optimised such that the enzyme reaction mixture was acidified with IM HCl to stop the reaction. TMB turned yellow with an absorbance maximum at 450 nm, and the absorbance was read at this, which was within the range of the Tecan Sunrise Remote EIA Reader available in the laboratory. This increased the sensitivity of the test 2-3 fold compared to the stop solution provided by the manufacturer which yielded blue end product that can be read at 650 nm [144].

Procedures in this study took into account the effects of the matrix and incubation time as found in the study of Huang et al. [99] . Matrix effects were controlled by using the same matrix (urine) for the calibrators, controls and case samples and the effect of the incubation time was solved by using automated procedures.

According to the manufacturer's product insert, tests performed using buffer as matrix produced about 20% higher sensitivity than those performed in urine. In this study the comparison was tested using spiked ketamine at 25 ng/mL in buffer (n=3) and urine (n=3); with a blank buffer and a blank urine included and the results were found to be similar to the manufacturer's findings. This was in agreement with another study which found that, when using the Neogen® kit for urine samples, the lower concentration ketamine standards generated higher B/B_0 (%) values compared to the same standard concentration prepared in buffer. Conversely, the authors of this study also observed that for higher concentration ketamine standards, the B/B_0(%) was lower than for same standard concentration prepared in buffer [99].

They also noticed that the results from calibration standards placed at the end of the microplate wells (shorter incubation time) apparently gave higher ketamine concentration than calibration standards placed at the beginning of the microplate wells, indicating that the shorter incubation time resulted in higher ketamine concentration readings [99] .

The ELISA test was also very specific to ketamine and showed a very low cross-reactivity with norketamine (2.1%) and no cross-reactivity at all to fourteen other commonly abused drugs tested in this study. The study of the Huang group from Taiwan found that the kit showed some cross-reactivity to the ketamine metabolite, dehydronorketamine (DHNK). The percent cross-reactivity decreased as the concentration of DHNK increased and was measured as 0.30, 0.18, 0.12, 007 and 0.05% at concentrations of 200, 500, 1000, 2000 and 4000 ng/ml, respectively [99]. However, this metabolite was not available in this study to confirm or refute these findings.

It was also claimed by the manufacturer that the kit has very low cross-reactivity (<0.01 %) to more than 70 drugs tested by them. Ketamine is a basic drug (P_{Ka} = 7.5) and can be metabolized into conjugates during phase II of drug

metabolism before being excreted in the urine. Due to low cross-reactivity to the metabolites, false negatives might be obtained with some case samples with higher concentrations of the main metabolite norketamine, dehydronorketamine or their conjugates, which are often present at higher concentrations than ketamine itself.

The ketamine ELISA was quite a sensitive test as shown by the low LOD obtained, 5 ng/ml. This finding was in agreement with the manufacturer who claimed that the kit is intended for use in the determination of trace quantities of ketamine in human urine, blood or oral fluid and suitable for forensic use. It was concluded that, if the test gave good correlation with the LC-MS/MS confirmation analyses, it would be reliable for use as a routine preliminary test for ketamine screening in a forensic laboratory.

The test had a wide linear range from 25 to 500 ng/ml which was good for capturing a range of concentrations in real case samples in forensic toxicology testing. At the cut off (25 ng/ml), 34 samples from the Malaysian Royal Police tested positive, showing that the kit can detect ketamine concentrations in abuse situations. 10 negative control samples from colleagues tested negative, showing that the kit can differentiate samples with no ketamine present. However the results could not be definitely accepted until tested by the confirmatory method.

4.7 Conclusions

A simple, rapid and efficient ELISA test for ketamine has been optimised and validated for the analysis of ketamine in urine samples. The Neogen® ELISA kit is adequately sensitive and specific for ketamine screening at a cut-off concentration of 25 ng/mL. The ELISA test has been shown to be highly specific to ketamine and demonstrated minimal (2.1%) cross-reactivity to its main metabolite norketamine. The kit demonstrated excellent precision for ketamine.

5 VALIDATION OF A METHOD FOR QUANTIFICATION OF KETAMINE AND NORKETAMINE IN URINE BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

5.1 Introduction

The aim of this study was to develop and validate an LC-ESI-MS-MS method for the quantification of ketamine and its major metabolite, norketamine, in urine. The other main metabolite dehydronorketamine was not included in this study due to no standard being commercially available at the time the study was conducted. General principles of HPLC and LC-MS have been described in Sections 3.21 and 3.22.

The method would then be applied to confirmatory analysis of thirty-four urine case samples from the Narcotics Department, Royal Malaysian Police that had been previously screened using the Neogen ketamine ELISA kits and qualitatively screened by GC-MS with a cut-off 350ng/mL at the Drug and Research Laboratory, Pathology Department, Kuala Lumpur General Hospital. The study would determine if the LC-ESI-MS/MS method showed good agreement with the ELISA method and if it could be used in future for routine analysis.

5.2 Operational Principle of LC-MS

A Thermo Finnigan LCQ™ Deca XP Plus was used for identification and quantification of analytes of interest in this thesis. The instrument has three main components: the HPLC system, electrospray interface (ESI) ionisation source and a mass spectrometer (the ion trap mass analyser) as shown in Figure 5-1.

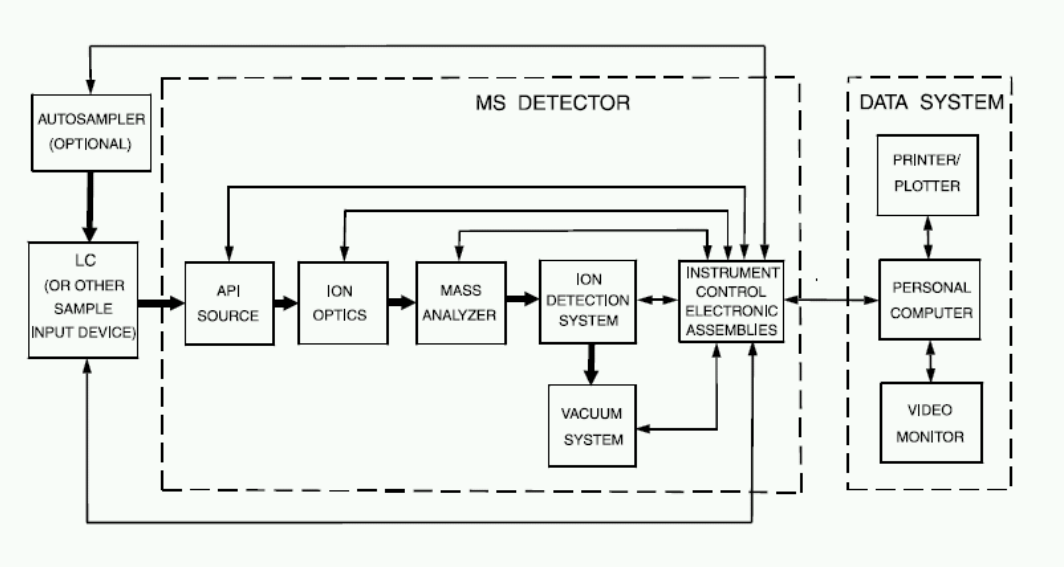


Figure 5-1 A block diagram of Thermo Finnigan LCQ™ Deca XP Plus System [119]

5.2.1 Electrospray Ionization (ESI) Interface

The ESI source consists of a needle and a series of skimmers (Figure 5.2). A skimmer produces a beam in which all the ions travel with exactly the same velocity so that their energies are proportional to their masses.

The LC eluent is introduced into an atmospheric-pressure ion source region through a fused-silica inner capillary which is inserted through the sample inlet and ESI needle. A sample solution is sprayed through the fine capillary needle into the spray chamber in front of the skimmer to form droplets.

The needle is supplied and held at a very high negative or positive voltage (3-5 RV) which causes charge transfer to the liquid. The droplets carry charge when they exit the capillary and, as the solvent evaporates, they decreases in size, ions come closer together and repel each other. The droplets split into microdroplets during the series of coulombic explosions which occurs when the repulsion overcomes the cohesive forces of the surface tension, leaving highly charged analyte molecules in the gas phase.

The charged molecules are transferred into the gas phase of the API stack (Figure 5.3) through an ion transfer capillary. Sheath gas and auxiliary gas consisting of nitrogen are also directed at the droplets to assist in nebulising the sample, stabilize the ion signal and help lower the humidity in the ion source during the analysis. The charged molecules travelling from the ion transfer

capillary will then enter the tube lens to be focused towards the opening of the skimmer. From the skimmer cone, the ions are directed into the ion optic assembly which separates the ions according to their mass to charge ratios (m/z), before entering the ion trap mass analyser.

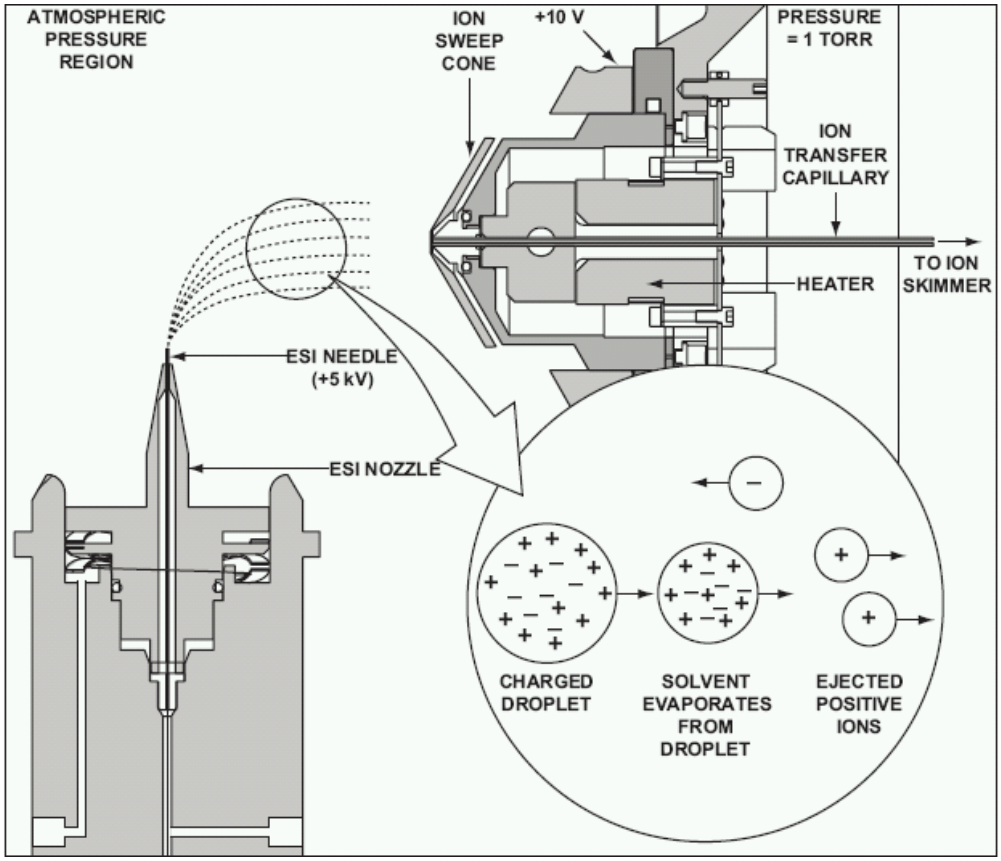


Figure 5-2 Schematic Process of ESI [119]

The equipment requires tuning for several MS detector parameters such as electrospray voltage, ion transfer capillary temperature, and tube lens offset voltage, capillary voltage, sheath and auxiliary gas flow for each analyte to achieve the optimise sensitivity of the analysis.

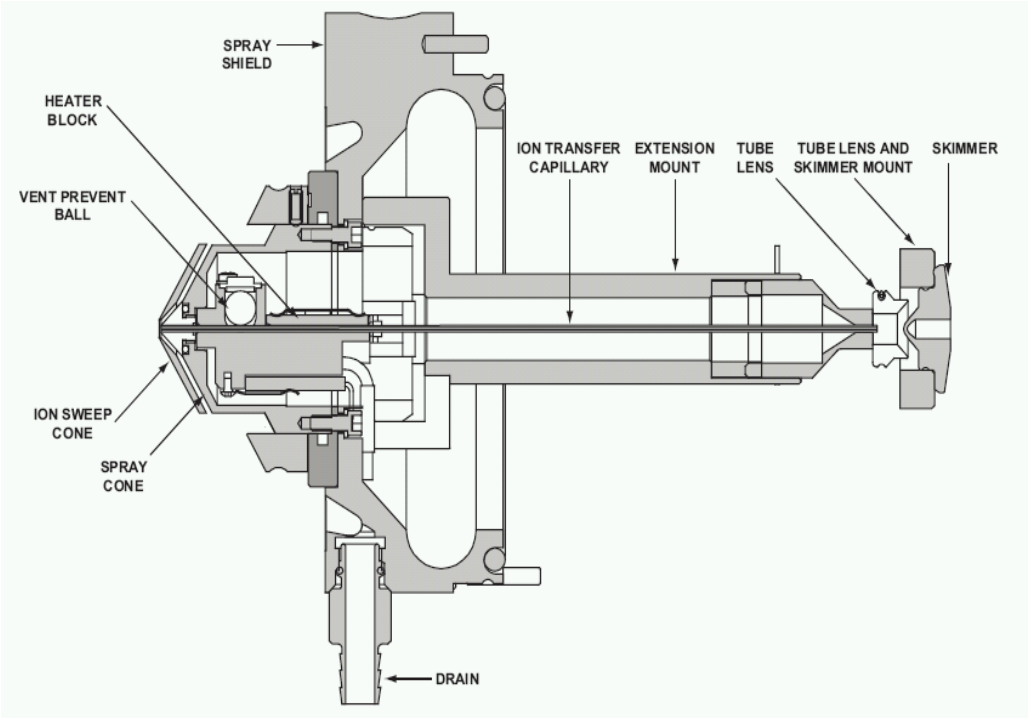


Figure 5-3 Cross-sectional view of an API stack [119]

In electrospray processes, the ions observed may be ions created by the addition of a proton (a hydrogen ion) and denoted $[M + H]^+$, or the addition of another cation such as sodium ion, $[M + Na]^+$, or the removal of a proton, $[M-H]^-$. Multiply-charged ions such as $[M + 2H]^{2+}$ may also be observed, especially with large molecules such as proteins. The schematic process of ESI ionization is shown in Figure 5-2 and the ionization mode and the ion type are presented in Table 5-1.

Table 5-1 Typical ions produced by ESI ionisation

Ionization mode:	Type of ions
Positive mode:	$[M+H]^+$ protonated molecule
	$[M+Na]^+$, $[M+K]^+$ … adducts
	$[M+CH_3CN+H]^+$ protonated molecule + solvent adduct
Negative mode:	[M-H] - deprotonated molecule
	[M+HCOO -] $^-$, … adducts

5.2.2 The Ion Optics

In the ion optic assembly, the ions will be separated according to their mass to charge (m/z) ratios [119]. The Deca XP ion optic consists of a quadrupole and octapole which are separated by the inter-octapole lens. The quadrupole and octapole are arrays of rods that act as ion transmission devices. An RF and DC offset voltage are applied to the rods to give rise to electric fields that guide the ion along the axis of quadrupole/octapole and allow only ions of a certain m/z ratio to pass into the mass analyzer.

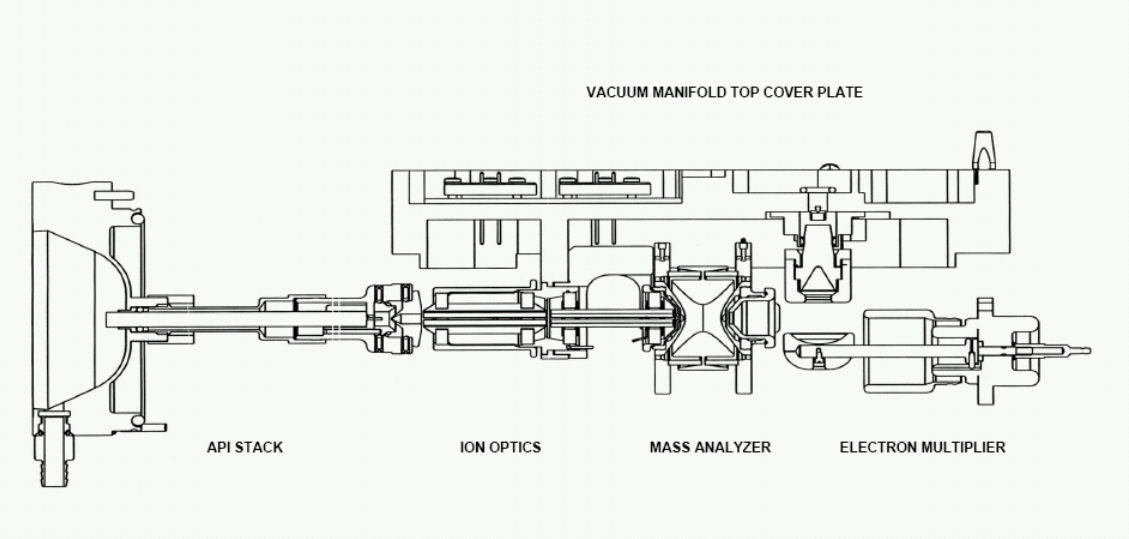


Figure 5-4 Cross sectional view of the LCQ Deca Plus ion optics [119]

5.2.3 The Ion Trap Mass Analyser

The ion-trap mass analyzer consists of three electrodes (one ring electrode and two end-cap electrodes) to trap ions in a small volume (Figure 5.5). The end-caps are separated either side of the ring electrode to form a hyperbolic cavity area which it is possible to trap (store) and analyse ions. The end-caps have small holes in the centre to permit the passage of ions into and out of the cavity. Ions which travel into the cavity will be trapped until a radiofrequency (RF) voltage is supplied to the ring electrode. The mass spectrum is obtained when the trajectories of ions of particular m/z values then become unstable and the ions are ejected through the exit lens to the detector.

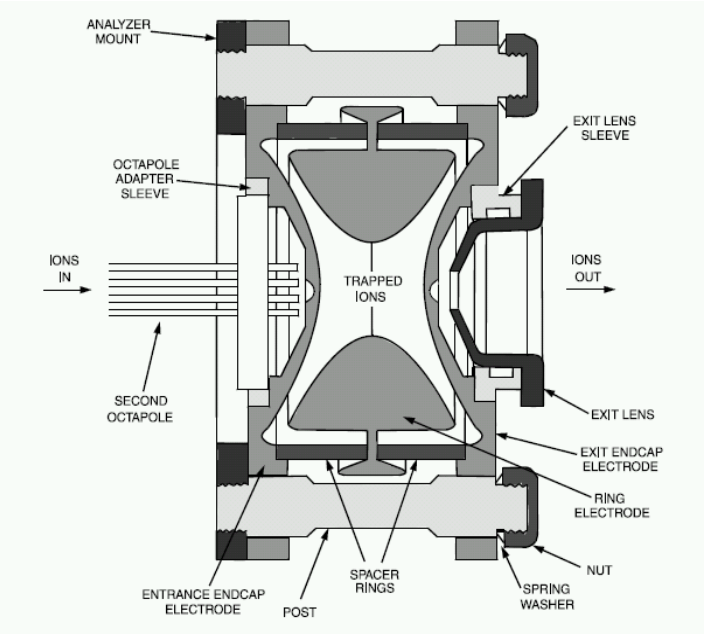


Figure 5-5 The ion trap mass analyser [119]

5.2.4 The Electron Multiplier Ion Detector

Electron multiplier tubes are similar in design to photomultiplier tubes. Their function is to multiply the ion current and they can be used in analog or digital mode. The electron multiplier consists of a series of biased dynodes that eject secondary electrons when they are struck by an ion. The secondary electrons are focused by the shape (curved surface) of the conversion dynode and are accelerated into the electron multiplier. If sufficient energy is provided by the electron multiplier cathode, the electrons will be ejected. In the detector, the ions will strike the surface of a conversion dynode to produce one or more secondary particles [119].

The secondary particles are focused by the curved surface of the conversion dynode and are accelerated into the electron multiplier. If the secondary particles have sufficient energy, the electron multiplier cathode will eject electrons and these electrons will strike the inner surface of the cathode to produce more electrons. Thus, a cascade of electrons is created, resulting in a measurable current. The current is converted to a voltage by an electrometer circuit and recorded by the MS data system.

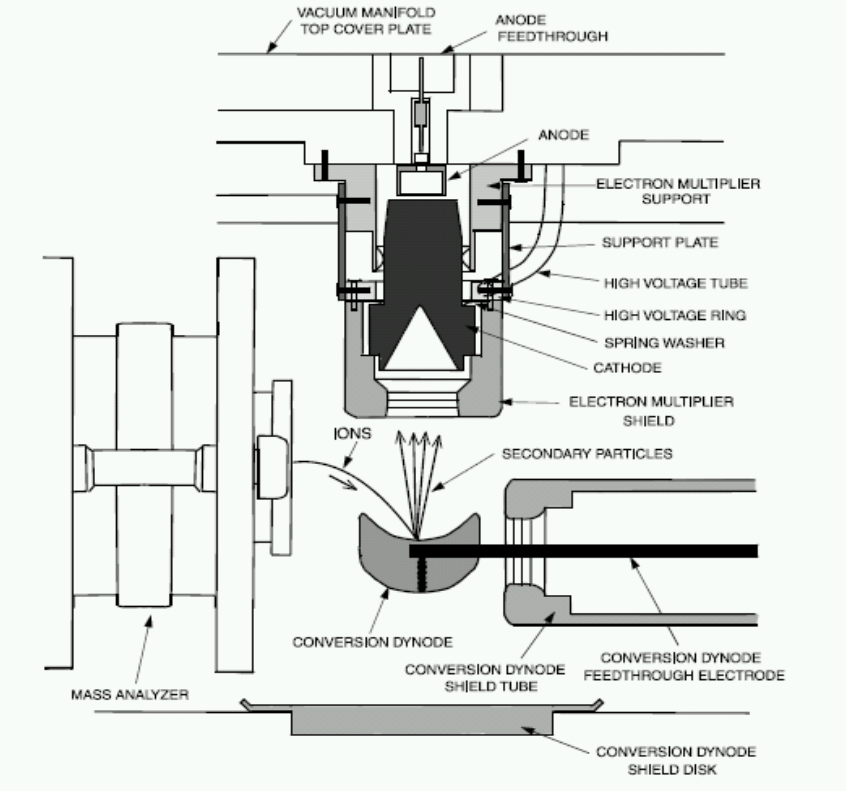


Figure 5-6 Cross sectional view of the ion detection system [119]

5.2.5 Tandem Mass Spectrometry

An instrument is called MS/MS or Tandem MS when the MS^n scan corresponds to two or more stages of mass analysis (*n* = 2 or more scan power). In an MS^n scan, parent ions are fragmented into product ions in one or more stages. Tandem mass spectrometry (MS/MS) is a more powerful technique to quantitate low levels of target compounds in the presence of a high sample matrix background compared to LC/MS.

Scan types include:

- full scan, which provides a full mass spectrum of each analyte or parent ion;
- Total Ion Current (TIC), in which each MS scan plotted as an intensity point;
- Selected Ion Monitoring (SIM), in which the MS is set to scan over a very small mass range, typically one mass unit, and only compounds with the selected mass are detected and plotted;
- Selected Reaction Monitoring (SRM), a two-stage (n = 2 scan power) technique in which parent ion and product ion pairs are monitored. In the first stage of

mass analysis, the ions formed in the ion source are stored in the mass analyzer. Ions of one mass-to-charge ratio (the parent ions) are selected and all other ions are ejected from the mass analyzer. Then the parent ions are excited so that they collide with background gas that is present in the mass analyzer. The collisions of the parent ions cause them to fragment to produce one or more product ions. In the second stage of mass analysis, the product ions are stored in the mass analyzer. Ions of one or more mass-to-charge ratios are selected and all other ions are ejected from the mass analyzer. Then, the selected ions are sequentially scanned out of the mass analyzer to produce an SRM product ion mass spectrum.

The main advantage of ion trap MS method, in addition to its compact size, is its ability to perform multiple stages of isolation and fragmentation of ions (MS^n), easily enabling the identification of unknowns. However, ion trap analysers cannot provide precursor scan and neutral loss modes of data acquisition. For quantification, ion trap analysers have been reported to be 10 times less sensitive when compared with the tandem quadrupole (triple quad) system operated in multiple reactions monitoring mode [145].

5.3 Experimental Section

5.3.1 Instrumentation

LC-MS-MS analysis of ketamine was carried out using a Thermo Finnigan LCQ Deca XP instrument (Thermo Finnigan, San Jose CA, USA) equipped with a Surveyor autosampler (AS 3000) and two pumps. The instrument was also attached to a computer system running Xcalibur 1.3 for data acquisition and processing.

Chromatographic separation was performed using a Synergi Hydro RP column (150 mm x 2.0 mm ID, 4 µm particle size) together with a 4.0 mm x 2.0 mm, 4 µm particle size guard column with the same packing material as the column (Phenomenex, Torrance CA, USA). The guard column holder was from Phenomenex, Macclesfield, UK. World Wide Clean Screen® SPE columns (ZSDAU 020) were purchased from United Chemical Technologies, Inc. (Pennsylvania,

USA). Gas-tight autosampler vials and caps were obtained from Kinesis Ltd, (Greensbury Farm Bolnhurst, UK).

5.3.2 Drug Standards and Chemicals

Ketamine, norketamine, ketamine-d4 and norketamine–d4 were obtained from Promochem (Teddington, UK). Ammonium acetate and ammonium formate were purchased from Fluka (Buchs, Switzerland). Formic acid, methanol and acetonitrile were HPLC grade, from BDH (Poole, UK). Ammonium formate was purchased from Sigma-Aldrich (Dorset, UK).

5.3.3 Stock and Calibration Standards

Working solutions with concentrations of 1 µg/mL and 0.1 µg/mL were prepared and used to prepare spiked samples for method validation, including seven point calibration curves at concentrations of 0, 50, 100, 200, 400, 800 and 1200 ng/mL. Stock and working solutions had a nominal shelf life of 6 months and were kept refrigerated (4 °C) when not in use and replaced on an as-needed basis.

5.3.4 Preparation of Solutions

5.3.4.1 Mobile Phase

An acidic mobile phase consisting of 3 mM ammonium formate and 0.001% formic acid in water was prepared by adding 0.189 g ammonium formate and 10 µl of concentrated formic acid to a 1000 mL volumetric flask and making up to 1000 mL with deionised water.

5.3.4.2 Phosphate Buffer (0.1 M, pH 5.0)

1.70 g of sodium hydrogen phosphate and 12.14 g of sodium dihydrogen phosphate were weighed out and transferred to a 1 L volumetric flask. 800 mL distilled water was added to the flask. The contents were dissolved by stirring and the pH was adjusted to pH 5.0 using orthophosphoric acid. The volume was made up to 1 L with deionised water. The buffer was kept in the refrigerator (4° C).

5.3.4.3 Sodium Acetate Buffer (1 M, pH 5.0)

42.9 g of sodium acetate trihydrate was weighed out and 10.4 mL concentrated glacial acetic acid (17 M) was measured and both were added to a 500 mL volumetric flask. 400 mL distilled water was added to the flask. The contents were dissolved by stirring and the pH was adjusted to pH 5.0 using 1 M acetic acid. The solution of was made up to volume with deionised water then kept in the refrigerator (4 °C).

5.3.4.4 Methanol with 2% Aqueous Ammonium Hydroxide

98 mL methanol was measured using a volumetric cylinder and 2 mL ammonium hydroxide was added to make up the volume to 100 mL. The solution was transferred into a bottle and was kept at room temperature for 2 months.

5.3.5 Urine Samples

Thirty-four urine samples which had previously screened positive using GC-MS were obtained from the Narcotics Department, Malaysian Royal Police collected from people arrested by the police at nightclubs and ten negative samples were obtained from volunteers in the laboratory. The positive samples were freeze dried and were kept in the freezer prior to analysis. All samples were screened by an optimised and validated ELISA method as described in Chapter 4.0.

5.3.6 Hydrolysis Method

All samples were hydrolysed enzymatically using ß-glucuronidase crude solution (Helix Pomatia). 1 mL urine or diluted urine sample was put into a tube. Dilution was done by adding 900 µl 0.1 M phosphate buffer pH 5.0 to 100 µl urine samples and was applied for those samples that were screened > 125 ng/mL by the ELISA method. 1 mL 1.0 M acetate buffer pH 5.0 was added into each tube followed by 40 µl ß-glucuronidase. All tubes were centrifuged and capped. The tubes were placed in an oven for 3 hours at 60° C. After cooling, 3 mL 0.1 M phosphate buffer pH 5.0 was added to each tube and was vortex mixed. The pH was adjusted to pH 5.0 with 1 M potassium hydroxide. The tubes were centrifuged at 2500 rpm for ten minutes. After centrifuging, the samples were ready for loading in the extraction method.

5.3.7 Extraction Method

Solid phase extraction (SPE) was used for method development and World Wide Monitoring Clean Screen® columns (ZSDAU 020) were selected for use in this study. The column size was 10 ml, with 200 mg sorbent. The columns were conditioned sequentially with 3 mL of methanol, 3 mL of distilled water and 1 mL 0.1 M phosphate buffer, pH 5.0.

The columns were loaded with urine samples which were allowed to drip through without any vacuum. Upon completion of sample loading, 3 ml of 0.1 M phosphate buffer pH 5.0 followed by 1 mL of 1.0 M acetic acid were applied to the columns to wash out interfering substances. The columns were then dried for 5 min under full vacuum. The analytes of interest were eluted into collection tubes using 2 mL of methanol containing 2 % aqueous ammonium hydroxide. The tubes containing the elution solvent were placed in drying blocks at 40 ± 0.5°C and the elution solvent was evaporated to dryness under a stream of nitrogen. The extracts were reconstituted in 150 µL of mobile phase in its initial composition and were vortex mixed. 20 µL of the reconstituted extract was injected for analysis.

5.3.8 LC-MS-MS Conditions

LC analysis was carried out using a mobile phase containing 3 mM ammonium formate + 0.001% formic acid and acetonitrile at a flow rate of 0.25 mL min^{-1}. The elution program consisted of a linear gradient over 13 minutes of 3 mM ammonium formate (95-5%). The percentage of ammonium formate was then decreased to 74 % between 13 to 22 minutes. It was held at 20 % between 22 to 29 minutes before being increased to the initial condition (95 %) between 30 to 36 minutes. 20 µl samples were injected on the Synergi Hydro column.

All mass spectral data were acquired in electrospray positive ion mode. The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimized for each analyte (Table 5.2 in the Results, Section 5.4). The probe voltage used was 4.5 kV. Internal standard data was collected in selected ion monitoring (SIM) mode and analytes were identified on the basis of their

retention time and full MS-MS spectra. The product ion ratios were monitored to gain further qualitative identification data.

5.3.9 Method Validation

5.3.9.1 Method Validation

Parameters investigated for method validation were limit of detection (LOD), lower limit of quantification (LLOQ), linearity, recovery, intra- and inter-day precision, accuracy and matrix effects. Validation procedures were mostly based on a paper published by Peters et al. [28].

5.3.9.2 Linearity, LODs and LLOQs

Urine calibration standards were prepared by spiking the appropriate amount of ketamine and norketamine stock solutions (1.0 µg/mL) into test tubes containing drug-free urine to provide final concentrations of ketamine and norketamine ranging from 0-1200 ng/mL. These were extracted using the SPE procedure describe earlier and analysed by LC-MS-MS. Calibration curves were constructed by plotting the peak area ratios of standards/internal standards against the spiked analyte concentrations and were subjected to linear regression analysis. Two replicate analyses were performed for each concentration to evaluate linearity for statistical purposes. LODs were calculated using Equations 5-1 and 5-2 where Y_B is the intercept, S_B is the standard error of the regression line and m is the gradient.

$$Y_{LOD}=Y_B + 3S_B \qquad \textbf{Equation 5-1}$$

$$LOD=(Y_{LOD}-Y_B)/m \qquad \textbf{Equation 5-2}$$

LOQs were calculated using the same method but using 10 times the standard error of the regression line.

$$Y_{LOQ}=Y_B + 10S_B \qquad \textbf{Equation 5-3}$$

$$LOQ=(Y_{LOQ}-Y_B)/m \qquad \textbf{Equation 5-4}$$

5.3.9.3 Precision and Accuracy

The intra- and inter-day precisions were determined by analysing extracts of human urine spiked with analytes at concentrations of 50, 500 and 1000 ng/mL five times in the same day (intra-day precision) and once a day during 5 successive days (inter-day precision). No commercially-available control standards were available and accuracy was determined by comparing the mean calculated concentration of the spiked urine samples with the target concentration.

5.3.9.4 Matrix Effect Assessment

This study was conducted to assess the interference caused by urine matrix components during LC-MS-MS analysis. Six replicates of 1.0 ml blank urine were spiked with 50 ng/mL, 500 ng/mL and 1000 ng/mL of ketamine and norketamine and compared with another six replicates which were spiked with the same concentration of ketamine and norketamine in 2 ml of loading buffer. All of the samples were vortex mixed, centrifuged and extracted using the described SPE procedure. 100 ng/mL of internal standard was added after the extraction. The percentage matrix effect was calculated according to Equation 5-5, where 'a' and 'b' are the peak area ratios of the analyte to internal standard in neat solution and human urine, respectively.

Percentage matrix effect = b/a x 100% **- Equation 5-5**

A value of <100 % indicates signal suppression whereas >100 % indicates signal enhancement due to matrix interference.

5.3.9.5 Recovery Studies

Ketamine and norketamine were spiked in 1.0 ml aliquots of drug free urine at concentrations of 50, 500 and 1000 ng/ml (n=5) and extracted using the SPE procedure. Two unextracted standards were also prepared at each concentration without internal standards and were kept in the fridge throughout the extraction. Internal standards ketamine-d4 and norketamine-d4 (100 ng of each)

were added to each tube before blowing down the samples under nitrogen. The extracted and unextracted standards were analysed by LC-MS-MS. Recoveries were determined by comparing peak area ratios of extracted standards/internal standards with those of unextracted standards, expressed as a percentage.

5.4 Results

5.4.1.1 Mass Spectral Characteristics

In this study LC-tandem mass spectrometry (LC-MS/MS) was used to provide selectivity, sensitivity and reliable results. Identification of ketamine and norketamine was based on retention time and full MS/MS spectra. Selected reaction monitoring (SRM) was used for data collection where one parent ion and two daughter ions were selected for each analyte and this fulfilled the requirements of the European Union for identification and confirmation of illicit drugs[146]. The major product ions with 100% relative abundance were used as quantification ions and the ratios of the other product ions provided qualification data. Parent ions were present at 10 % of the intensity of the quantification ions.

The mass spectral data acquired for both analytes and their deuterated internal standards were based on electrospray (ESI) positive ion mode which produced protonated molecular ions, $[M + H]^+$. The precursor ion for ketamine was at m/z 238 and the daughter ions were at m/z 220, 207 and 179, while the norketamine precursor ion was at m/z 224 and the daughter ions were at m/z 207, 206 and 179. Precursor ions for ketamine-d4 and norketamine-d4 were at m/z 242 and 228 respectively and their daughter ions were at m/z 224, 211 and 183 for ketamine-d4 and m/z 211, 183 and 129 for norketamine-d4.

Specimen SRM chromatograms from collision-induced dissociation of parent ions at m/z 238 for ketamine and m/z 224 for norketamine and MS-MS spectra are shown in Figure 5-7(blank), Figure 5.8 (5ng extracted ketamine and norketamine) and Figure 5.9 (positive sample).

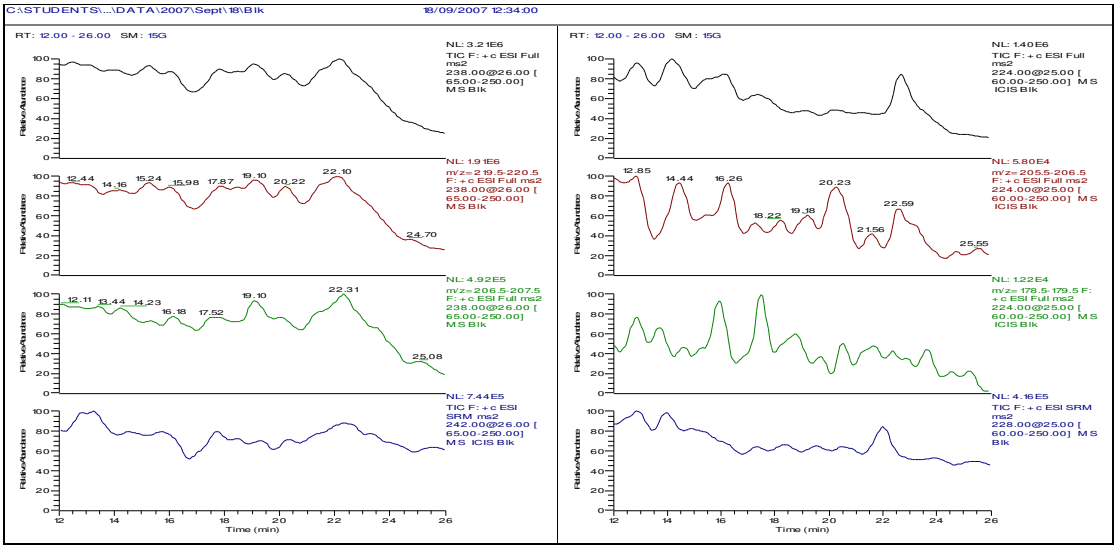


Figure 5-7 SRM chromatograms from collision-induced dissociation of parent ions at m/z 238 for ketamine and m/z 224 for norketamine and MS-MS spectra showing the quantitation ions of 220 for ketamine and 207 for norketamine in a blank sample

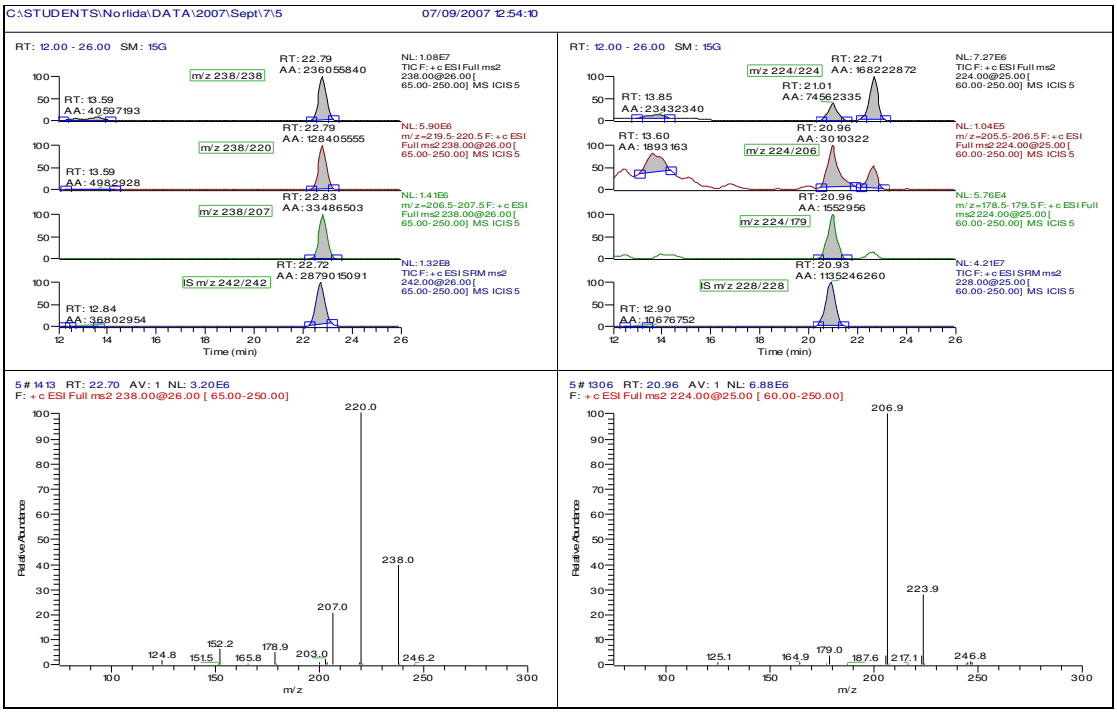


Figure 5-8 SRM chromatograms from collision-induced dissociation of parent ions at m/z 238 for ketamine and m/z 224 for norketamine and MS-MS spectra showing the quantitation ions of 220 for ketamine and 207 for norketamine in a 5 ng/mL ketamine and norketamine standard extracted from urine

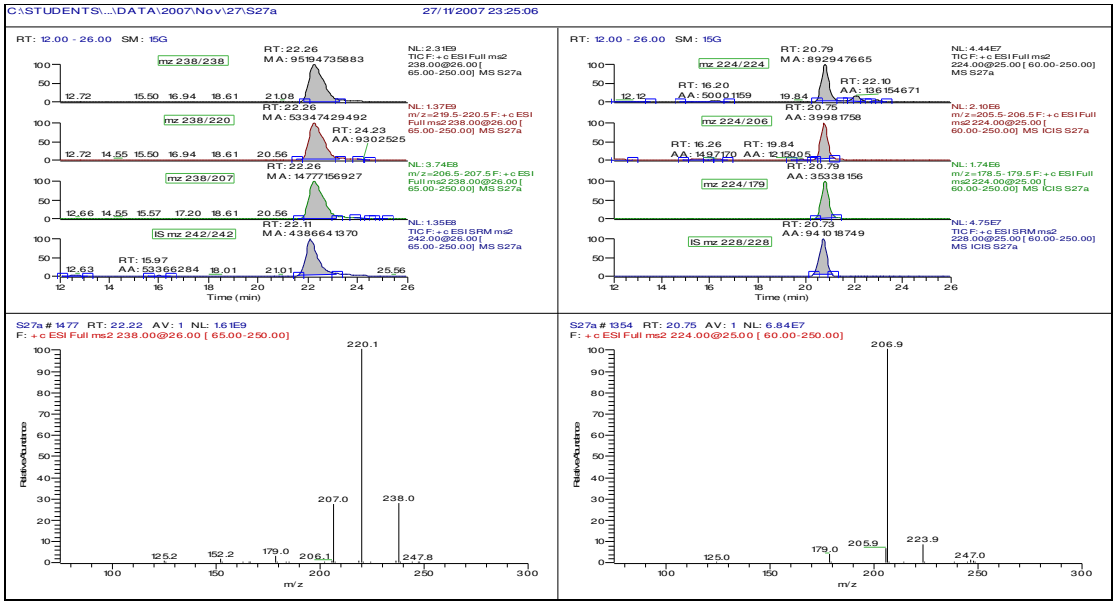


Figure 5-9 SRM chromatograms from collision-induced dissociation of parent ions at m/z 238 for ketamine and m/z 224 for norketamine and MS-MS spectra showing the quantitative ions of 220 for ketamine and 207 for norketamine in (a positive sample for ketamine (17260 ng/mL) and norketamine (1040 ng/mL)

Subsequent validation procedures and analysis of real case samples were carried out using the above parent and daughter ions. The optimum collision energy, precursor and product quantitation and qualifier ions are shown in Table 5-2.

Table 5-2 MS-MS parameters for Ketamine, Norketamine, Ketamine-d4 and Norketamine-d4 in the ESI positive mode

Compound	Precursor Ion (m/z)	Product Ions (m/z)	Collision Energy (%)
Ketamine	238	220*, 207, 179	26
Norketamine	224	207*, 206, 179	25
Ketamine-d4	242	224*, 211, 183	26
Norketamine-d4	228	211*, 183, 129	25

*** Quantitation ions (100% relative abundance)**

5.4.1.2 Linearity, LOD and LLOQ

The linearity of the LC–ESI-MS-MS method was evaluated within the range 2–1200 ng/mL. Linear correlation coefficients (R^2) of the calibration curves were 0.9995 and 0.9979 for ketamine and norketamine, respectively (n=3), as shown in Figure 5.10.

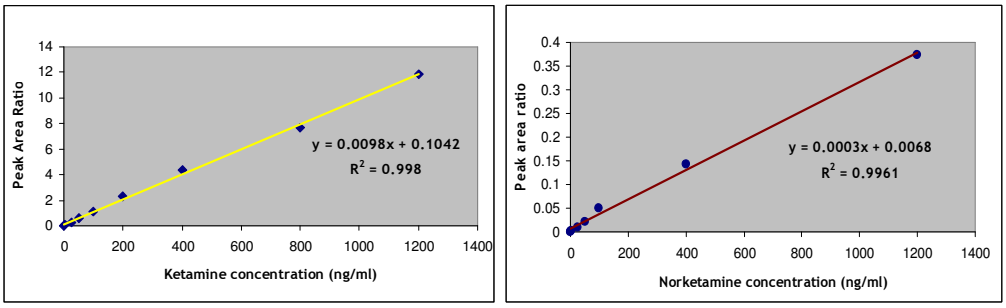


Figure 5-10 Calibration curves for ketamine and norketamine in urine over the concentration range 0– 1200 ng/mL.

The linearity measurement provided the necessary information to estimate the limit of detection based on linear regression analysis. The LODs for ketamine and norketamine were 0.56 and 0.63 ng/mL respectively and LOQs were 1.9 and 2.1 ng/mL respectively. The linearity, the linear equation, limit of detection and limits of quantification are summarised in Table 5-3.

Table 5-3 Analytical characteristics of LC–ESI-MS-MS method for ketamine and norketamine in urine

Compound	Linear range (ng mL^{-1})	Linear equation	Correlation coefficient (R^2)	Detection limit (ng /mL)	Quantitation limit (ng/ mL)
Ketamine	0-1200	Y = 0082x + 0.08	0.9995	0.6	1.9
Norketamine	0–1200	Y = 0.0004x + 0003	0.9979	0.6	2.1

5.4.1.3 Intra- and Inter-day Precision and Accuracy

The intra-day precision and accuracy were evaluated by performing five replicate analyses of three different concentration spiked QC samples in the same day. The precision of the method was calculated as the relative standard

deviation (RSD) of the results. Inter-day precision and accuracy were determined by analysing similar spiked QC samples over a period of 5 days, with fresh calibration curves being prepared in duplicate analyses each day. Intra- and inter-day precisions are shown in Table 5-4.

Accuracies were determined by comparing the mean calculated concentration of the spiked urine samples with the target concentration. The intra- and inter-day accuracies for all the samples were from 96.6 % to 105.2%. The results indicated the repeatability of the assay was acceptable and demonstrated high accuracies.

Table 5-4 Intra- and inter-day precision and accuracy of the QC samples of ketamine and norketamine in urine

Analyte	Spiked concentration (ng mL^{-1})	Intra-day (n=5)			Inter-day (n=5) x 5 different days		
		Mean ± S.D.	Accuracy (%)	R.S.D. (%)	Mean ± S.D.	Accuracy (%)	R.S.D. (%)
Ketamine	50.0	49.0 ± 0.8	98.0	1.7	49.8 ± 2.5	99.6	5.0
	500.0	499 ± 6.2	99.8	1.3	498± 3.9	99.6	0.8
	1000.0	997 ± 7.4	99.7	0.7	996 ± 5.0	99.6	2.2
Norketamine	50.0	47.4 ± 3.3	94.80	7.0	48.0 ± 1.7	95.9	3.6
	500.0	497 ± 4.5	99.47	0.9	498 ± 2.6	99.6	0.5
	1000.0	997 ± 0.5	99.70	0.5	995 ± 2.7	99.5	0.3

5.4.1.4 Assessment of Matrix Effects

When a sample contains high concentrations of salts or an excess of another analyte that can ionise under the operating conditions, there might be a competition effect in the ionisation. This is called a matrix effect. Most researchers include the assessment of matrix effects in their LC-MS method development to ensure that the chromatographic separations developed using different matrices were not affected or showed minimal or acceptable matrix effects, at least when performing quantitative analysis [32].

The results show that matrix effects caused by co-extracted interferences in the urine were small and were acceptable for both ketamine and the metabolite

norketamine. Matrix effect analysis for ketamine showed ion suppression of <10 % while norketamine showed ion enhancement of <20 % at three concentration levels. A summary of the observed matrix effects is given in Table 5-5.

Table 5-5 Matrix effects during LC-MS-MS analysis of ketamine and norketamine in urine (n=3)

Analyte	Spiked concentration (ng mL^{-1})	Matrix Effect (%) [% RSD] n=6
Ketamine	50	-8.6 [2.3]
	500	-4.7 [10.4]
	1000	-4.0 [5.1]
Norketamine	50	+19.7 [13.2]
	500	+ 6.3 [11.9]
	1000	+12.9 [10.7]

5.4.1.5 Recovery Studies

Recoveries of ketamine and norketamine from human urine samples at low (50 ng/ml), medium (500 ng/ml) and high (1000 ng/ml) concentrations are presented in Table 5-6. The method produced high recoveries ranging from 97.9 to 113.4 % with RSDs of less than 10%.

Table 5-6 Recoveries of ketamine and norketamine from human urine samples

Analyte	Spiked concentration (ng mL^{-1})	Recovery (%) (n=5)	SD	RSD (%)
Ketamine	50	113.4	0.1	1.1
	500	107.6	0.6	3.6
	1000	98.3	1.8	7.1
Norketamine	50	102.1	0.1	1.1
	500	104.3	0.5	5.6
	1000	97.9	0.3	3.8

5.4.1.6 Application to Urine Samples

The results for blank samples included in each analysis indicated no ketamine and norketamine present. A set of 34 specimens was analysed using the optimised and validated method along with calibration standards with concentrations ranging from 0 to 1200 ng/ml of ketamine and norketamine. Three quality control samples at 50 ng/ml, 500 ng/ml and 1000 ng/ml were included to check the performance of the analysis. Samples were diluted 10 times with phosphate buffer pH 5.0 and reanalysed if the ketamine and norketamine values did not fall within the set calibration concentration range. Samples which still contained very high ketamine and norketamine concentration after the second analysis were subject to further dilution (100 times).

For the LC–MS–MS analysis in this study, a concentration equal to or more than the high control (1000 ng/mL) is defined as a high concentration. All of the 34 samples were confirmed positive by LC–MS/MS with most of them containing high concentrations of ketamine and norketamine. The data showed no consistent ratio of norketamine to ketamine but in most samples the concentration of norketamine appeared to be higher than that of ketamine. The average ratio was 14.2 and the ratio median was 4.3. The ratio of norketamine to ketamine ranged from 0.001 to 82.2 with norketamine being higher than ketamine in 27 out of 34 samples. The LC-MS/MS results demonstrated approximately the same results as ELISA for the parent drug ketamine as presented in Table 5-7.

In this study, referring to the field of toxicology, a urine specimen was considered positive by the LC–MS/MS method if either the ketamine or norketamine concentration was higher than its LLOQ. The results also highlighted that there is a ketamine abuse problem in Malaysia as all 34 samples were confirmed positive and showed ketamine and norketamine present in high concentrations.

Table 5-7 Results of analysis of case urine samples by GC-MS, ELISA and LC-MS-MS.

Sample Number	GC-MS qualitative screening at cut off of 350 ng/mL	ELISA Concentration (ng/mL)	LC-MS/MS Ketamine (ng/mL)	LC-MS/MS Norketamine (ng/mL)	Ratio of Norketamine: Ketamine
1	Positive	>125	*2830	*6960	2.5
2	Positive	>125	880	*3970	4.5
3	Positive	>125	*2670	*6800	2.5
4	Positive	>125	*1200	*1230	1.0
5	Positive	>125	*5340	*4070	0.8
6	Positive	>125	*1210	*6960	5.7
7	Positive	>125	*4570	420	0.1
8	Positive	>125	920	*6400	6.9
9	Positive	>125	150	1830	12.1
10	Positive	>125	200	*10990	56.0
11	Positive	>125	790	*5250	6.6
12	Positive	32	30	690	22.2
13	Positive	>125	*11070	*8810	0.8
14	Positive	37.4	40	*1540	42.9
15	Positive	25.8	20	*1080	49.2
16	Positive	29.6	30	*1250	46.4
17	Positive	>125	*8460	220	0.03
18	Positive	>125	130	*8980	71.3
19	Positive	>125	*1150	*10320	8.9
20	Positive	>125	560	*8650	15.4
21	Positive	>125	130	*10680	82.2
22	Positive	>125	790	*3270	4.1
23	Positive	>125	30	740	22.5
24	Positive	>125	120	*1020	8.3
25	Positive	>125	130	250	1.9
26	Positive	>125	190	200	1.1
27	Positive	>125	*17,260	*1040	0.06
28	Positive	>125	*2610	*3254	1.2
29	Positive	>125	*1810	*2200	1.2
30	Positive	>125	*2360	*3800	1.6
31	Positive	>125	*8150	*1000	0.1
32	Positive	>125	218	*1000	4.6
33	Positive	>125	*31670	25	0.001
34	Positive	>125	*4560	*5620	1.2

*** High concentration equal to or more than the highest quality control sample, 1000 ng/mL**

5.5 Discussion

Human urine samples used in this study were collected by the Royal Malaysian Police from persons in bars in the southern part of Peninsular Malaysia. Urine is one of the preferred matrices for control of drug abuse and the only legally recognised matrix for workplace testing in many countries. Urine is considered advantageous because of the presence of compounds and their metabolites as well as because the volume of sample available is higher than with other matrices, while the collection is non-invasive.

According to Wieber et al. [147], only very small percentages of unchanged ketamine (2.3 %), norketamine (1.6 %) and dehydronorketamine (16.2 %) are eliminated in urine compared to 80% of dose which is present as conjugates of hydroxylated metabolites of ketamine. This study was aimed at the detection of total ketamine and norketamine in urine samples, though LC-MS-MS allows detection of conjugates, including glucuronides [148]. This was because no standards of ketamine metabolite glucuronides were commercially available during this study.

The LC-MS/MS method was intended to be used for confirmation purposes and applied an enzymatic hydrolysis step to cleave glucuronide conjugates of hydroxylated metabolites. In this study it was thought that the hydrolysis would not make any difference to the total ketamine and norketamine concentrations, since neither ketamine nor norketamine has a hydroxyl group and they could only form glucuronides on the amine nitrogen atom (N-glucuronides), on the oxygen atom of the ketone in its enol form or on a carbon atom, which would be very unusual. However, there are no published reports of N-glucuronidation of ketamine and norketamine and it is possible that the hydrolysis step could be omitted. The hydrolysis step would improve the sensitivity of the method (i.e. percentage of true positives detected) if the hydroxylated metabolites were targeted, but this was not possible since standards were not available. Nevertheless, the LODs and LLOQs obtained for ketamine and norketamine were very low, 0.6 ng/mL and LLOQ 1.9 and 2.1 respectively, which are suitable for determination of trace concentrations of ketamine in urine in forensic toxicology.

This is supported by previous studies, for example, Kronstrand et al. [148] in their analysis of buprenorphine, norbuprenorphine and their glucuronides by LC-MS/MS concluded that direct LC-MS/MS analysis without hydrolysis and pre concentration is suitable only for screening but the method with SPE increased the sensitivity for both analytes from 20 µg/L to 1 µg/L. Another study by Al Asmari et al. [149] confirmed that a hydrolysis procedure was effective in cleaving glucuronide conjugates in the range of concentrations encountered in urine cases in his study which indicated buprenorphine abuse. This finding indicates that the hydrolysis method used in this study could be effective in indicating ketamine abuse when the case samples are analysed. However, the direct method without hydrolysis would be considered in future work because of the routine heavy workload in the author's laboratory in Malaysia.

This study applied solid phase extraction for the extraction of ketamine and norketamine. World Wide Monitoring Clean Screen® columns have been used in many studies for detection of drugs and have shown good recoveries [150, 151]. The solid-phase extraction method used operated by a mixed-mode cationic exchange mechanism based on the sorbent composition of C8 chains and benzenesulfonic acid residues. As a result, ketamine and its metabolites can be retained on the column via both hydrophobic and ionic interactions and high recoveries of more than 97 % have been obtained for ketamine and norketamine at concentrations of 50 ng/ml, 500 ng/ml and 1000 ng/ml. The SPE step also helped in obtaining low limits of detection and in reducing the matrix effects during LC-MS-MS analysis [151].

For the urine sample analysis, ketamine and norketamine were both detected by LC–MS/MS in all 34 of the urine specimens. However, the concentrations of ketamine and norketamine in each sample varied widely, 22-31670 ng/mL for ketamine and 25-10990 ng/mL for norketamine. This could be caused by a number of factors such as dose and route of administration of ketamine, the time interval between administration and collection of the urine sample, the subject's rate of metabolism, weight and health. The duration of the drug in the body and severity of effects varies from one person to another [95]. Most results for ketamine were in the range 51–10,000 ng/mL with a median of 903 and most

norketamine results were in the range 1001-10,000 ng/mL with a median of 2720, as shown in Figure 5-9.

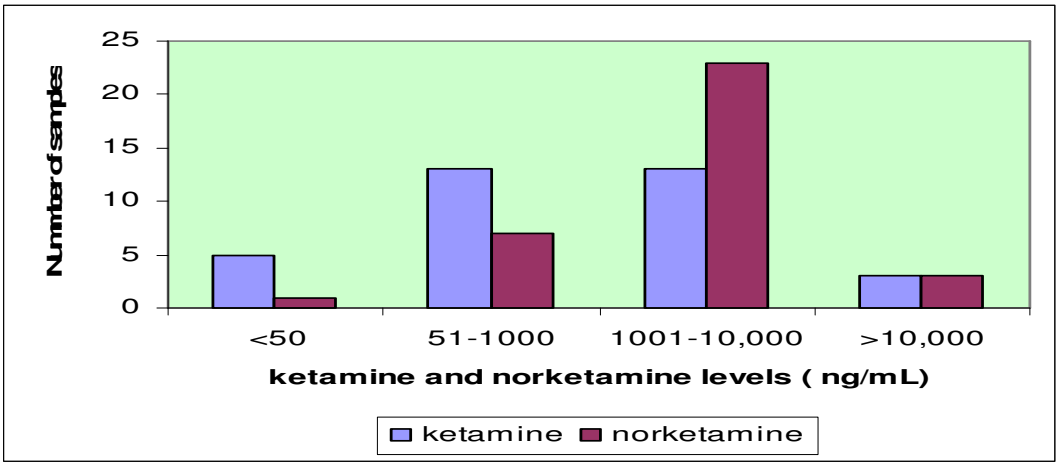


Figure 5-11 LC-MS/MS results for urine samples

Wieber and co-workers reported that the $t_{½}$ of ketamine in urine was 3.37 ± 0.14 h, the $t_{½}$ of NK was 4.21 ± 0.35 h, with both analytes being undetectable after 22 h, and the $t_{½}$ of DHNK was 7.21 ± 1.39 h, which was undetectable after 60 h. It seems that those samples which had ketamine concentrations greater than those of norketamine in this study were probably collected soon after use of the drug. The high concentrations of ketamine and norketamine in most samples may suggest regular intake of ketamine in those abusers. The high concentrations of ketamine and norketamine in real samples collected from participants in bars show the same pattern of abuse in Malaysia and Taiwan [99] and alarmed the governments that ketamine was emerging as a new drug of abuse in those countries.

As part of the present study, a comparison was made of the LC–MS/MS and ELISA methods for urine specimens. The linear range of the Neogen® ELISA kit for ketamine was evaluated as 25–500 ng/mL. A previously published paper comparing ketamine ELISA with GC-MS [99], using 100 ng/mL as the ELISA cut- off, as well as a GC-MS cut-off of ≤ 2.6 ng/ml demonstrated a poor correlation between methods (less than 30%) in 43 samples. In another study [106], 90.9 % sensitivity and 98.9% specificity and 1.1% false positive results were found when using 10 ng/mL of ketamine as the ELISA screening cut-off and 15 ng/mL of ketamine as the GC–MS confirmation cut-off.

Based on the previous studies above and the results for ELISA method evaluation, 25 ng/mL an intermediate value between 10 ng/mL[99] and 100 ng/mL[106] was chosen as the ELISA cut-off for screening and the LLOQs of ketamine and norketamine (2 ng/ml) as the LC-MS/MS cut-offs. Comparison of the two methods yielded 100% sensitivity and specificity with no false positive or false negative results, as presented in Table 5-8.

The method also found that there was no ketamine or norketamine in the blank samples. The test performance of the two methods indicated that the cutoff value determined for this assay, 25 ng/mL, was shown to be fit for purpose and had no norketamine cross-reactivity issues at the levels that were detected for these particular samples. Therefore the combination of the ELISA screening test and an LC–MS/MS confirmation analysis produced an acceptable, highly sensitive, specific and efficient system for the determination of ketamine and norketamine in urine samples.

Table 5-8 ELISA and LC-MS-MS test performance

Test results				Test Performance				
Screening ELISA at cut –off 25 ng/mL		Confirmation LC–MS/MS		Efficiency (%)	Sensitivity (%)	Specificity (%)	FP rate (%)	FN rate (%)
Positive	Negative	Positive	Negative	100	100	100	0	0
34	10	34	10					

Efficiency = (TP + TN) × 100/Total.
Sensitivity = TP × 100/(TP + FN).
Specificity = TN × 100/(TN + FP).
False-positive rate = FP × 100/(FP + TN).
False-negative rate = FN × 100/(TP + FN).

5.6 Conclusions

A liquid chromatography-tandem mass spectrometric method for the confirmation and quantitation of ketamine and norketamine in human urine has been developed and validated. The method demonstrated good linearity, LOD, LOQ, accuracy and precision and had acceptable matrix effects. The efficiency of ELISA as a screening method has been evaluated using the LC-MS/MS method.

Thirty four urine specimens collected from suspected drug users were used and the results were well correlated with those obtained using the validated LC–MS/MS method as the confirmation procedure. These methods complemented each other and showed that both ELISA and LC-MS/MS methods are sensitive and specific for determination of ketamine abuse based on analysis of urine samples. The data demonstrated no false positives or negatives at a cut-off 25 ng/ml for ELISA and at the LOQ values of ketamine and norketamine for LC-MS/MS confirmation.

A combination of ELISA and LC–MS/MS methods can be reliably used as routine tests in a dual approach test strategy (screening and confirmation) for the determination of ketamine in urine specimens and could in principle be applied to other biological samples. This study also found that ketamine and norketamine were present in all specimens, with more than 90 % of the samples having high concentrations, and highlighted that there is a ketamine abuse problem in Malaysia.

6 MOLECULARLY IMPRINTED POLYMERS (MIP$_S$)

6.1 What are MIPs?

Molecular imprinting is defined as the ability of one molecule to attach to another molecule that has a complementary shape. Molecular imprinting produces a polymer that displays molecular recognition for a chosen analyte. The polymer is synthesised in the presence of the analyte, which acts as a print molecule, template or mould. Polymerisation occurs in the presence of functional monomers, excess cross-linking agent, a non-aqueous solvent and an initiator.

After the synthesis, the template is removed by an extraction process, leaving behind imprinted binding sites (cavities) within the polymer network that are tailored in size, shape and chemical functionality to the template. A schematic diagram of the polymerisation is shown in Figure 6-1. Under appropriate conditions, these cavities are able to rebind with the template molecule or structurally related compounds in a strong and selective manner when they are reintroduced to the polymer.

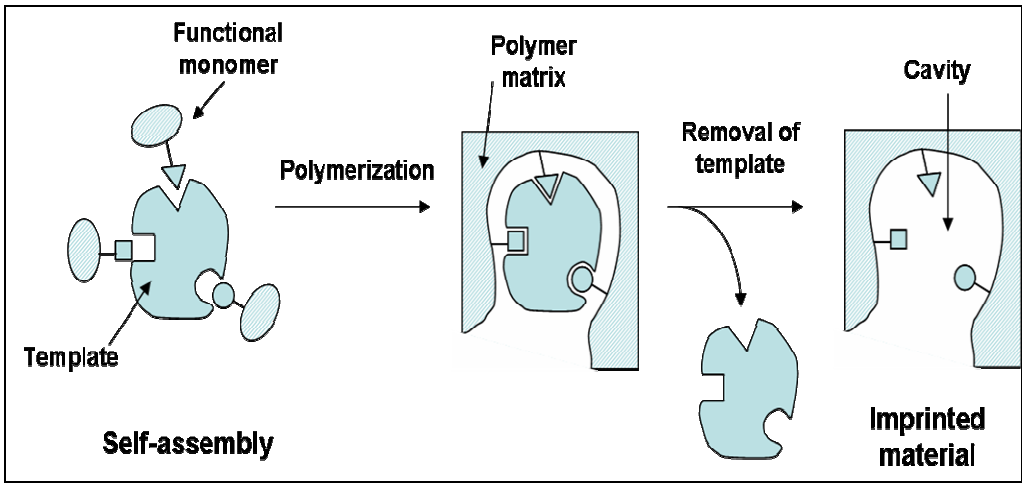


Figure 6-1 Polymerisation steps in the synthesis of a molecularly imprinted polymer

MIPs are highly cross-linked polymers that have pre-determined selectivity for a single analyte or a group of structurally related analytes. The materials are physically stable, resistant to mechanical stress, high pressure and elevated

temperatures. Furthermore, MIPs possess an imprint memory of more than 100 times without loss of memory. These characteristics make a MIP an ideal analytical material [131, 152, 153].

The synthesis of MIPs usually involves a parallel process involving synthesis of a non-imprinted polymer (NIP) under conditions identical to those of the MIP except that the template is absent. In principle, the NIP is entirely analogous to the MIP except that any binding sites within its porous structure are non-selective. The NIP can therefore be used as a benchmark for assessing the selectivity of the MIP such as recovery and breakthrough as reported in published papers [154,155].

6.2 History of Molecular Imprinting

Today the technology of molecular imprinting is a growing area in many fields with broadened approaches and applications. MIP technology has been intensely explored as shown by the number of publications on MIP over 70 years from 1930 to 2003 in Figure 6-2. There was a dramatic increase in the number of publications over recent years from 1995 onwards, with more than 2000 scientific papers. The research done so far included the characteristics, the design and the applications of molecular imprinting polymers in separations sciences, biosensors, synthetic antibodies, catalysts and nanotechnology.

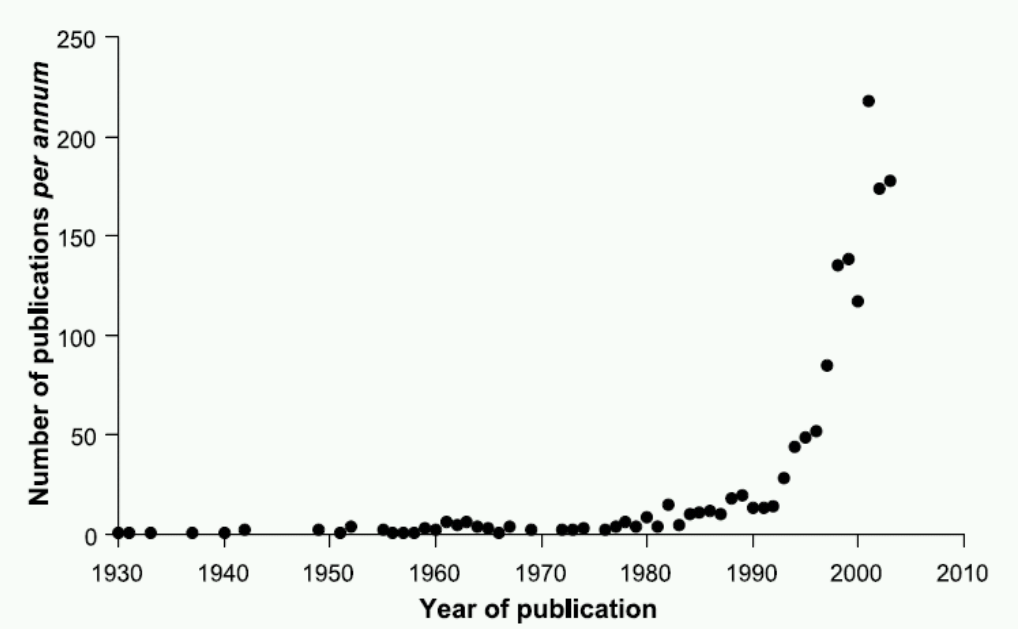


Figure 6-2 The number of publications of MIP per annum from 1931-2003

Molecular imprinting was first reported in 1931 by a Soviet chemist, Polyakov and co-workers from Kiev. They imprinted various additives such as benzene, toluene and xylene (templates) in a silica matrix formed from sodium silicate, water and ammonium carbonate as the gelating agent during the drying process. The results showed that the imprinted additives had selectivity effects for use in chromatography [156].

In the 1940's Linus Pauling hypothesized that a process similar to molecular imprinting could be responsible for the selectivity of antibodies to their respective antigens [157]. Later in 1949, the theory was supported by experiments done by his graduate student, Frank Dickey, who demonstrated imprinting and specific adsorption of several different dyes in silica using methyl orange as the template. The approach done was slightly different from Polyakov because the template was present from the beginning and some organic acids were used as acidifying agent. The method demonstrated selectivity for the dye. His technique has contributed to the present MIP development methodology [158].

Imprinting in silica gained some popularity over the next 15 years, but did not really catch on due to the instability of the silica matrix, high variability in the absorption capacities of the matrices between batches and limitations in the diversity of compatible template molecules [159].

With the decline of silica imprinting, molecular imprinting in organic polymers was introduced. The concept of molecular imprinting was revived in the 1970's when Wulff and Klotz discovered that highly crosslinked organic polymers could be used to make molecular imprints with high specificity. They successfully prepared a molecularly imprinted synthetic organic polymer capable of discriminating between the enantiomers of glyceric acid using covalent approaches [160]. In covalent imprinting the monomer and the template are bound by a covalent linkage, then the covalent conjugate is polymerised under conditions where the covalent linkage is intact, the template is removed by cleaving of the covalent linkage and the analyte rebinding is also formed under the same covalent linkage.

Ten years later in the early 1980s, Mosbach and co-workers introduced a non-covalent approach which has been widely applied in modern imprinting work [161]. In non-covalent imprinting the monomer and the template are connected by non-covalent interactions; after polymerisation the template is removed by certain solvents and rebinding of analytes occurs through the same non-covalent interactions.

In 1995, Whitcombe et al. reported an intermediate approach that appeared to combine the advantages of both approaches; the polymer was prepared by covalent linkage but the analyte rebinding was formed by the non-covalent interactions[162] . Details of covalent, non-covalent and semi-covalent imprinting are explained in Section 6.4. Imprinted polymers have now been used to capture a wide range of compounds, such as drugs and steroids and today some research procedures enable the capture of large and fragile proteins [163,164].

6.3 Principles of Molecular Imprinting

The success of the imprinting experiment relies on the careful choice of the template molecule, the functional monomers, the cross-linking agent, the polymerization solvent, called the porogen, and the radical initiator. MIP is usually synthesised for an intended analytical use, for example, as a sorbent in an SPE column, that implies the choice of a given template molecule.

The structure and the functionalities of this molecule define the subsequent properties of the binding sites. Even though molecular imprinting synthesis is simple and easy to perform, it depends on criteria such as cost, availability of a template and the interactions sites.

Normally, expensive or difficult-to-synthesize templates can be substituted by a structural analogue to decrease the cost of the material. Some researchers use a structural analogue [165,166,167] or an isotope (atoms of the same chemical element which have a different number of neutrons) [168,169] as an alternative imprint molecule, which is known as "dummy imprinting" as shown in Figure 6-3 for sildenafil [170]. This process should give rise to imprints that have the ability to bind the target analyte. In this case template leaching from the polymer will not lead to erroneous results in the analysis of the target analyte.

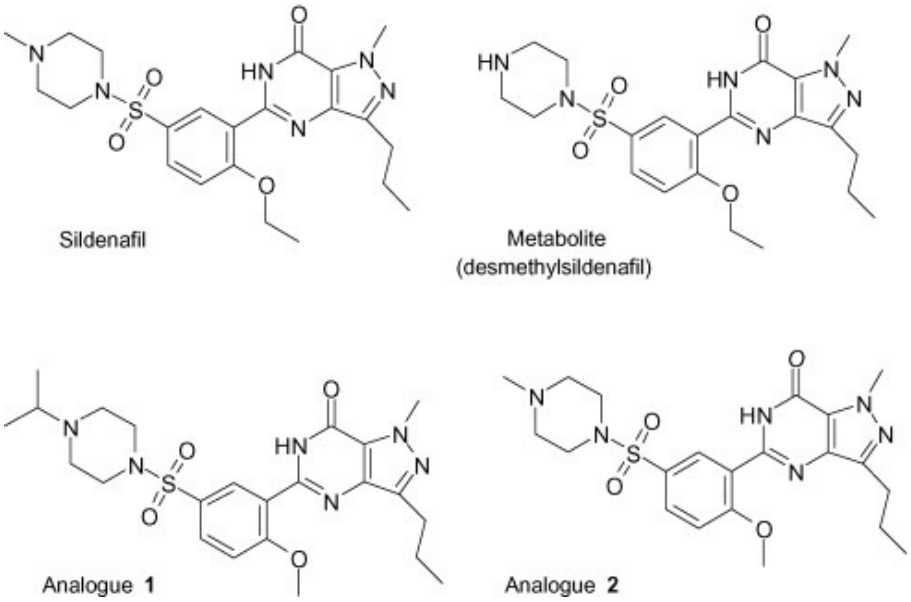


Figure 6-3 Examples of analogues of sildenafil used as templates in MIP synthesis

6.4 Approaches to MIP Synthesis

There are two basic approaches to molecular imprinting. The first one is the pre-organised approach developed by Wulff and co-workers [171] in which reversible covalent bonds maintain the aggregates in solution prior to polymerisation. The other is the self-assembly approach developed by the Mosbach group [172] in which the pre-arrangement between the print molecule and the functional monomers is formed by non-covalent or metal coordination interactions (Figure 6-4).

Both imprinting procedures make use of a high percentage of crosslinker resulting in rigid and insoluble polymers. These approaches used template-assisted assembly and thus can be performed in a very direct way, leading to an artificial recognition matrix. Covalent, non-covalent and semi-covalent linkage methods of synthesising MIPs are based on these two basic approaches.

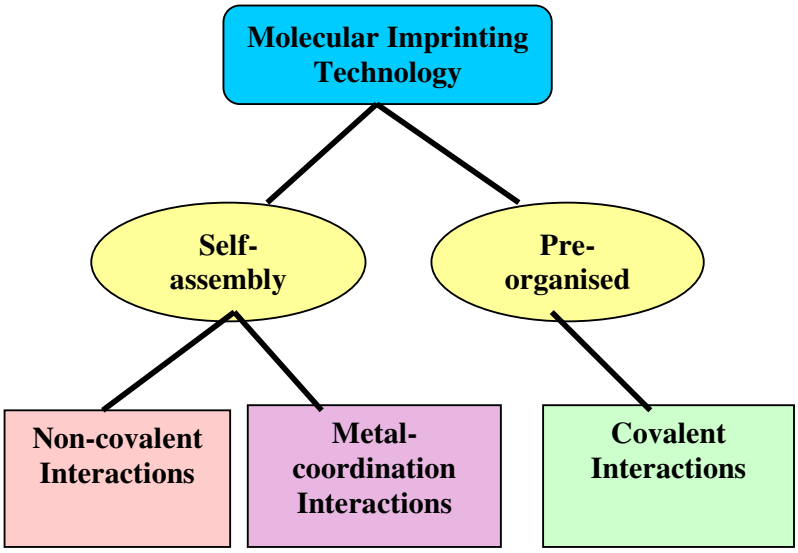


Figure 6-4 Molecular imprinting technology

In covalent imprinting, templates and monomers are bound to each other by covalent linkages. Polymerisation occurs under conditions where the covalent linkage is intact. After polymerization, the templates are removed by cleaving the bonds and the functionality in the cavities is able to rebind with target molecules by the same covalent interactions. The main advantages of this technique are that the monomer/template complexes are stable and stoichiometric and that a wide variety of polymerization conditions can be employed. Slow release and binding of templates and less economical synthesis of monomer/template complexes limit its application.

In non-covalent imprinting, the complex of template and functional monomer is formed *in situ* by non-covalent interactions, such as hydrogen bonding, electrostatic forces, van der Waals forces, or hydrophobic interactions. Templates are removed by disrupting the interactions and extracting the template using appropriate solvents. Rebinding of analytes by the polymer also occurs through the same non-covalent interactions.

This technique is advantageous in terms of easy preparation of the template/monomer complex, easy removal of the templates from the polymers and fast binding of templates to MIPs. Non-covalent imprinting is the most widely used for the preparation of MIPs. In this technique, optimisation is needed and the polymerization conditions must be carefully chosen to minimize

non-specific binding sites in the formation of the labile complex of template and monomer.

Semi-covalent imprinting is a hybrid approach of covalent and non-covalent imprinting. Covalent bonds are used in the polymerisation steps and non-covalent interactions are used for the re-binding steps of analytes of interest. Semi-covalent imprinting combines the main advantages of covalent and non-covalent binding techniques - the stable and stoichiometric complex in covalent imprinting and the fast guest binding (rapid kinetic rebinding of the target analytes) in non-covalent imprinting. Therefore some workers combined both techniques in their research. The types of imprinting linkages are shown in Figure 6-5.

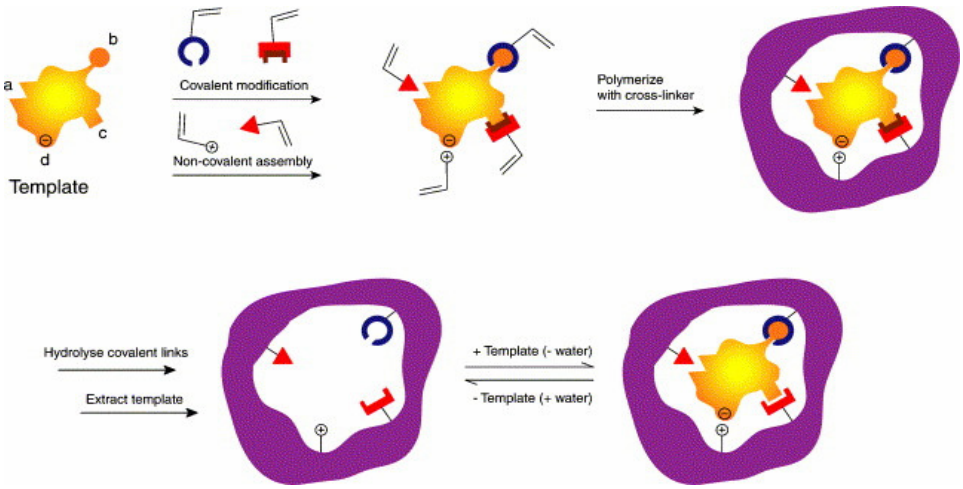


Figure 6-5 Types of binding during molecular imprinting [173].

Covalent and non-covalent molecular imprintings have the advantages and disadvantages summarised in Table 6-1. Based on the advantages, non-covalent imprinting shows more versatility than covalent imprinting.

Table 6-1 Advantages and disadvantages of covalent and non-covalent imprinting [174]

Characteristics	Covalent Imprinting	Non-Covalent Imprinting
Synthesis of monomer-template conjugate	necessary	unnecessary
Polymerisation conditions	rather free	restricted
Removal of template after polymerisation	difficult	easy
Guest binding and guest release	slow	fast
Structure of guest binding site	clearer	Less clear

6.4.1 Templates

Templates or targets are very important in molecular imprinting. The structure and functional groups of a template must be compatible with and able to attach to the functional groups of the monomers to define the subsequent properties of the binding sites. An ideal template must withstand polymerisation conditions which may involve mechanical stress, high pressure and elevated temperatures or UV radiation. A template must not be too expensive and must be available in the market in powder form. A selected template must produce a polymer that has a high affinity and rebinding selectivity to the analytes of interest when used in real applications such as for extraction of drugs in forensic toxicology.

Several of the drawbacks of MIPs can be overcome by the use of stoichiometrically associating monomer-template systems, which means that the relative amounts of monomer and template are calculated as a balanced chemical reaction to produce the percent yield of the imprinted polymer. The use of stoichiometric systems has resulted in a range of receptors exhibiting high capacity and effective recognition properties in aqueous media [175,176,177].

6.4.2 Functional Monomers

In non-covalent imprinting, the role of the monomers is to create the non-covalent binding interactions within the imprinted binding sites. To enhance this

process the monomers are used in excess compared to the templates, such as a 1:4 molar ratio. The excess of monomers helps in the formation of template-monomer complexes and maintains its integrity during the polymerisation [178].

Today, many monomers are synthesised using vinyl or acrylic groups as imprinting matrix and are commercially available. These monomers usually contain inhibitors or stabilizers (hydroquinone or phenols) to avoid undesired polymerisation during storage and therefore must be distilled before use. Typical functional monomers, shown in Figure 6-6, are carboxylic acids (acrylic acid, methacrylic acid, vinylbenzoic acid), sulphonic acids (acrylamido-methylpropanesulphonic acid) and heteroaromatic weak bases (vinylpyridine, vinylimidazole).

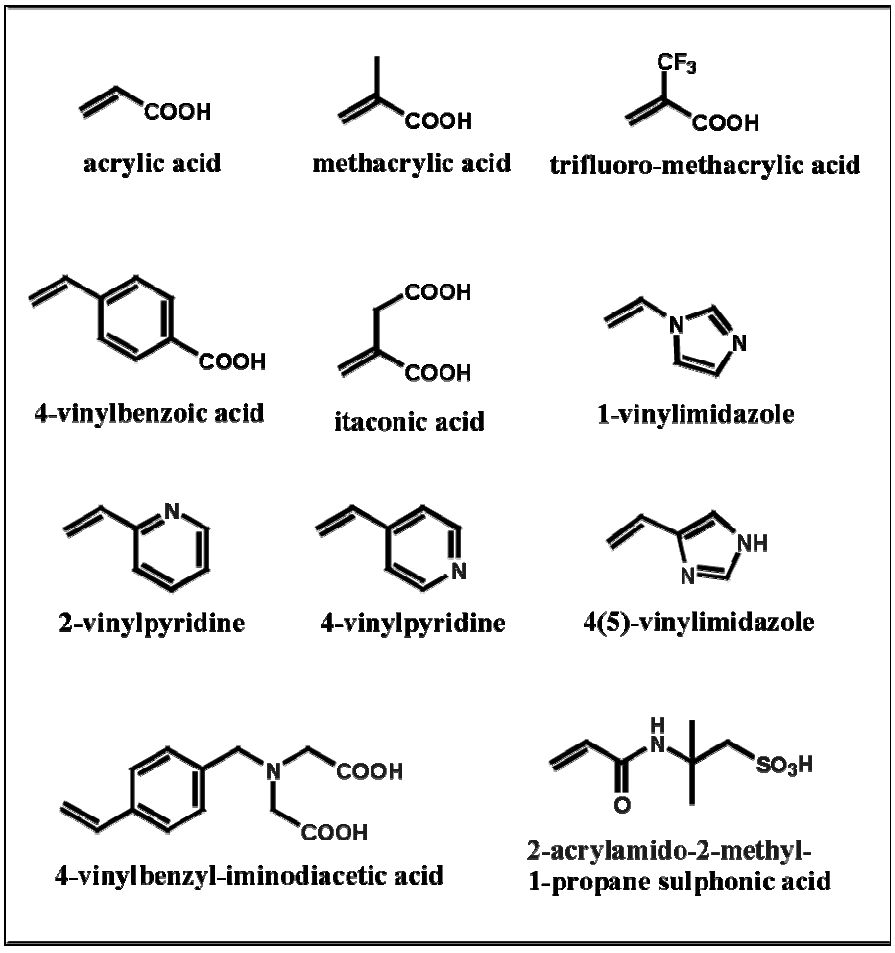


Figure 6-6 Some monomers used for the synthesis of MIPs.

6.4.3 Cross-linking Agents

In an imprinted polymer the cross-linker fulfils three major functions. First, both covalent and non-covalent binding use a high degree of cross-linking. Therefore they can control the morphology of the polymer matrix. Second, the role of the cross-linkers is to stabilise the imprinted binding site in the desired polymer structure. Third, the cross-linkers impart stability to the polymer matrix.

The reactivity of a cross linker and a monomer used in a polymerisation should be similar to avoid them being predominant over each other and copolymerisation not taking place sufficiently. The commonly utilised cross-linkers such as ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are shown in Figure 6-7. Standard formulations such as 1:4:20 of template, monomer and crosslinker have been proposed to give a simple, fast and rational way of obtaining MIPs with improved molecular recognition sites and in which polymers with cross-linker ratios in excess of 80% are often the norm [178, 179].

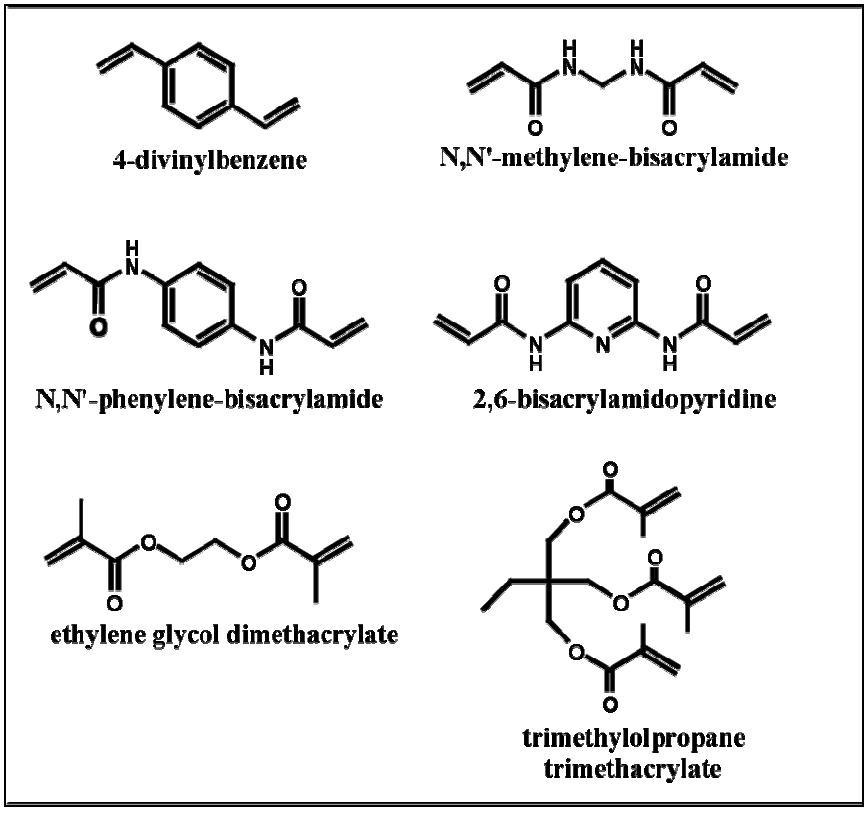


Figure 6-7 Different types of crosslinkers used in the synthesis of MIPs.

6.4.4 Porogens/solvents

An organic solvent has many roles in a polymerization reaction and is responsible for the outcome of a molecular imprinting process. One role is to dissolve the reagents for the polymerisation. The solvent also facilitates the template-monomer complex formation and thus enhances the imprinting efficiency.

The most important role of a solvent is to provide a porous structure in the polymer for promoting the rate of rebinding of analytes and removal of template. This is done by controlling the strength of non-covalent interactions and allowing the polymer chains to move apart and swell in a solvent. The spaces occupied by the solvent in the polymer become pores in the polymer after post-reaction treatment. A low surface area and a low macroporosity increase the difficulty in accessing the binding sites and may diminish recognition. Another role of the solvent is to disperse the heat generated during the reaction and therefore avoiding any side reactions occuring in the polymerisation [174].

In non-covalent imprinting the solvent polarity affects the recognition sites in a polymer in a proportional manner. Solvents with low dielectric constants (K), such as toluene and dichloromethane, can promote the strength of template-monomer binding and a fairly polar solvent such as acetonitrile (K = 36) leads to more macroporous polymers than chloroform (K = 5) [132].

6.4.5 Initiators

Free radical initiators such as 2,2'-azobisisobutyronitrile (AIBN) and 2,2'-azobis(2,4-dimethylvaleronitrile) (ADVN) are commonly used for polymerisation reactions. Due to their electrical neutrality, they contain an odd number of electrons and are highly reactive and can be used to initiate free radical polymerization and other radical reactions. In the polymerisation process, radicals formed by decomposition of the initiator (Figure 6-8) attack the monomer and produce propagating radicals.

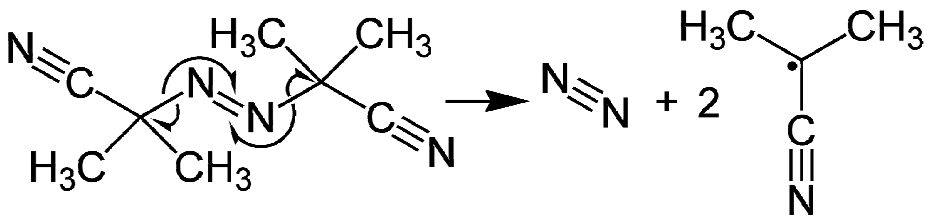


Figure 6-8 Decomposition of AIBN to form two 2-cyanoprop-2-yl radicals

One example of the conversion of methyl methacrylate monomer by AIBN in the initiation step in a radical polymerisation is illustrated below in Figure 6-9.

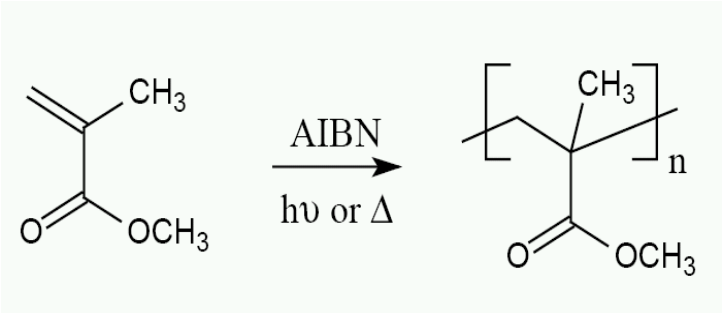


Figure 6-9 Conversion of methyl methacrylate monomer into poly (methyl methacrylate) using AIBN in free radical polymerisation

The imprinting process can be carried out under a wide range of conditions such as using solvents with different polarity and pH and this characteristic allows the polymerisation reaction to be optimised. Initiation of free radical polymerisation can occur via photolysis (UV) or thermolysis.

During the polymerisation process, it is important to remove molecular oxygen that can trap radicals in the reaction mixtures and retard or stop the polymerisation. This can be done by degassing with nitrogen and freeze-thaw cycles under reduced pressure.

6.5 MIP Application Areas

Molecular imprinting is a generic technology and can be used in a variety of applications such as chromatography, affinity solid phase extraction, selective permeable membranes, drug or fragrance release matrices, sensors and

multisensors for environmental and clinical diagnostics and as catalysts in chemical processes in industry [174,180]. To date, solid phase extraction using MIPs by far dominates the applications and some of the MIP-based separation products have been commercialised.

6.6 Molecularly Imprinted Solid Phase Extraction (MISPE)

MIPs are amongst the most selective phases used in SPE and this technique is known as molecularly imprinted solid-phase extraction (MISPE). MISPE is based on conventional SPE where the processes of conditioning, loading, clean-up and elution are typically applied. The use of MIPs as selective sorbents in SPE was first pioneered by Sellergren et al. in 1994, who successfully extracted pentamidine, a drug for treatment of AIDS-related pneumonia, from urine samples [129]. The continuing development of MISPE indicates its potential value in affinity separation studies due to the high separation factors and resolution performance obtained [179, 181,182].

Previously, most MISPE studies focused on the optimisation of polymer preparation and SPE procedures and the characteristics of the MIP materials. There are very few papers on the extraction of analytes from real samples. According to Feng and his group in their review, there have been about 110 original papers on MISPE applications to real samples published in the five years from 2001 t0 2006[128]. The distribution of the studies can be divided into biological samples (33.9 %), environmental samples (36.5 %), drug samples (14.8 %), food samples (12.2 %) and other samples (2.6 %). Out of these, none of the papers involved the field of forensic toxicology as in this thesis.

6.6.1 MISPE Protocol and Application

It is important to underline the fact that MIPs are not intrinsically selective. Therefore optimisation of the extraction steps is required, especially the washing and elution steps, in terms of pH, ionic strength and solvent composition. It is the MISPE procedure used (conditions of percolation, washing and elution) that confers selectivity on the MIP. Theoretically, the best results expected when using MIPSPE correspond to the achievement of a recovery close

to 100% on the MIP with no retention on the NIP after percolating real samples containing the target analytes.

Most published studies of MISPE have used an off-line mode mainly, due to its simple procedure and instrumentation required, which are similar to off-line SPE methods with conventional sorbents. The main advantages of this format are that the whole operation is simple and easy, that more solvents and additives can be used without regard to their influence on the subsequent chromatographic analysis, and that consequently a high enrichment factor and selectivity can be obtained.

Furthermore, in cases where an organic solvent is used as wash solvent, the MIP needs to be dried in order to overcome the immiscibility with aqueous systems during the loading step. This drying step is difficult to combine with an on-line procedure. In this study, a small amount of imprinted polymer (20 mg) was packed in SPE cartridges and these were taken through the typical SPE process in order to isolate the target compounds from real samples. The off-line mode also allowed the use of stronger elution conditions which were sometimes not compatible with the analytical instrumentation.

On the other hand, on-line MISPE involves the automation of MISPE, coupled with the analytical system. This mode minimises sample manipulation between the pre-concentration and analysis steps, thus reducing the loss of analytes and the risk of contamination as well as providing improved accuracy and reproducibility [183]. Because it has been automated, on-line MISPE can reduce the time for pre-treatment of real samples. Additionally, the sample volume needed can be smaller because the whole sample extract is transferred to the analytical column [184].

The MIP phase shows maximum selectivity when the experimental conditions are chosen that generate the selective interactions that are usually obtained in organic solvents (porogens) used for MIP synthesis. This approach allows the MIP to be used for trapping analytes from aqueous solution by hydrophobic or ionic interactions. Washing with a solvent breaks selective binding of matrix components and, finally, washing with an organic solvent disrupts the strong bonds between the polymer and target analytes.

As most MIPs are synthesized in nonpolar and aprotic solvents, MIPs seem to be well adapted to the clean-up of complex matrices. The direct extraction of compounds from aqueous matrices on MIPs is more difficult. During the percolation of an aqueous sample, retention of trace analytes is mainly ensured by non-selective hydrophobic interactions with the polymeric matrix. A careful choice of the washing solvent should be made; it should enhance the development of selective polar interactions between the analytes and the cavities. Thus, the use of a control polymer, a non-imprinted polymer (NIP), in parallel is essential to evaluate the real selectivity of the MIP and the contribution of non-specific interactions.

6.6.2 Advantages and Limitations of MISPE

There are several advantages of the MISPE technique. One of those is that MISPE can perform selective extraction to pre-concentrate and separate target analytes from complex matrices, which will lead to cleaner extracts and less interferences during analysis and subsequently lead to increased sensitivity.

It is well known that MISPE sorbents are very stable. They can be exposed to a broad range of organic solvents, can withstand high temperatures and can be used over broad pH ranges without loss of selectivity, which allows them to be stored at room temperature for prolonged periods of time. Another unique characteristic of MISPE is that it can reduce matrix effects in LC-MS by improving selectivity/sample cleanup when compared to conventional SPE. This is due to the tailor-made selectivity of the sorbent material created during the design and production of the MIP.

Conversely, there are few disadvantages of MISPE. Most of the time the template can be removed completely but template bleeding may cause false positives in the analysis of analytes in real samples. The use of an analogue molecule or dummy template which is structurally related to the target analyte can overcome this problem. For example a bromide molecule can be used instead of chlorinated template molecule. Andersson et al. reported the synthesis of an MIP using a template that was a close structural analogue of the target analyte, sameridine [185].

Some researchers have come out with several techniques of template removal including thermal annihilation, microwave-assisted extraction and desorption with supercritical fluids in cases where an analogue molecule is not available [186].

The problem of irregular size and shape of particles from monolith polymerisation may influence the effectiveness of the extraction. This drawback is overcome by several alternative techniques for preparing more homogeneous MIP particles such as precipitation, dispersion and suspension polymerisation [187,188,189].

The other disadvantage of MISPE is that each sorbent must be custom made in-house which may be laborious or costly. Nowadays this problem may be solved by using specialist commercial laboratories to prepare MIP materials or by buying from commercial suppliers that sell MISPE columns.

6.7 Aims

The work in this thesis involved the investigation of the potential of a molecularly imprinted polymer material in a MISPE application in tandem with LC-MS-MS analysis for drug determinations in forensic toxicology. The aims of the study were:

[1] Synthesis of anti-ketamine MIPs in collaboration with the Polymer Group, Pure and Applied Chemistry, University of Strathclyde, Scotland.

[2] Optimisation of a MISPE technique using the anti-ketamine MIP and application to real samples.

[3] Investigation of the potential of commercial MISPE columns such as the Amphetamine SupelMIP from Supelco for extraction of other matrices such as blood and urine.

7 SYNTHESIS OF MOLECULARLY IMPRINTED POLYMERS BASED ON A KETAMINE TEMPLATE

7.1 Introduction

There is an on-going challenge for rapid and efficient determination of compounds with low cost analytical methods in the environmental, forensic and drug development fields. Methods developed using biological molecular recognition such as those using enzymes and antibodies are often time- and cost-consuming. Molecularly imprinted materials, which involve solid materials containing cavities that have shapes and functional groups complementary to the imprinted template molecule, can be synthesised and be used as alternatives to natural receptors [159]. MIPs as synthetic receptors can be used in many applications such as the separation, extraction and detection of drugs and their metabolites and therefore can be useful in the field of forensic toxicology.

From the beginning, MIPs have found their main applications in the fields of chromatography and solid phase extraction (SPE) for the separation, pre-concentration and detection of low molecular weight compounds, including drugs and their metabolites, from different matrices such as blood, plasma and urine samples [190,191,192,193]. The only problem that must be solved is template bleeding, which can interfere with drug detection and must be solved before MIPs are used for any analytical purpose.

Nowadays, with an increasing amount of research in MIP technology focusing on the characterization of MIPs and their applications, also with the help of computer simulations, there is some hope that the weakness of MIPs can be overcome. MIP technology has been commercialised, for example, one company focusses on MIP products for solid phase extraction (MIP4SPE®) and separation (MIP4LC® for HPLC) [194,195] .

In the past, there was no attempt to use MIP materials as sorbents for extraction of ketamine prior to further analysis or as stationary phases in liquid chromatography columns for detection of ketamine and its metabolite in the field of forensic toxicology. In this chapter the synthesis of MIP materials for

(R/S)-ketamine and the potent enantiomer (S)-ketamine and chromatographic evaluation of the MIPs will be described.

7.2 Aim

The aim of this study was to synthesise MIPs using free base *(R/S)-ketamine* and the single enantiomer *(S)-ketamine* as templates and to evaluate the imprinting factors using liquid chromatography before applying them to MISPE protocols.

7.3 Experimental Section

This study was carried out in collaboration with the Polymer Unit, Department of Pure and Applied Chemistry, University of Strathclyde, Scotland. The study used the expertise and facilities of the Polymer Group, University of Strathclyde and the MIPs prepared were used in forensic toxicology.

7.3.1 Chemicals and Materials

(R/S)–ketamine.HCl and (S)–ketamine.HCl were purchased from Sigma Aldrich (Dorset, UK). Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were obtained from Aldrich (Steinhem, Germany) and 2,2'-azobisisobutyronitrile (AIBN) was from Acros Organics (Geel, Belgium). Dichloromethane (DCM), sodium bicarbonate and anhydrous magnesium sulphate were from Sigma Aldrich (Dorset, UK). HPLC grade acetonitrile, acetone and toluene were purchased from BDH (Poole, UK).

7.3.2 Converting Ketamine.HCl Salts into Free Base Ketamine

For the synthesis of an anti-ketamine MIP, the template must be present as free base ketamine. Most commercial ketamine standards are sold as ketamine hydrochloride salt which must be converted to the free base form to serve as a template in the polymerization process. The process of obtaining free base ketamine was as follows:

(R/S)-ketamine salt (1.0 g) was partitioned between saturated aqueous sodium bicarbonate (20 mL) and DCM (20 mL). The DCM layer was separated from the

aqueous layer and then washed twice using sodium bicarbonate (2 x 20 mL) and once with deionized water (1 x 20 mL). The DCM layer was dried over anhydrous magnesium sulphate and the drying agent was removed by vacuum filtration. The DCM was removed by evaporating in a weighed round bottom flask using a rotary evaporator under reduced pressure.

The product was dried overnight at 40° C in a vacuum oven. The mass of the product was 0.87g (95.1%). The procedure was repeated to convert 1 gram of (S)-ketamine hydrochloride salt into the corresponding free base. The yield for (S)-ketamine free base was 0.85g (97.6 %).

To ensure the identity of the end products, the melting point was measured and found to be 92-93° C, which is the melting point of free base ketamine. The ^{1}H ^{1}H-NMR spectra were also recorded and were consistent with the structures of R/S- and S-ketamine free bases.

7.3.3 Preparation of Solvents for Synthesis of Ketamine MIPs

7.3.3.1 Porogen –Toluene

Analytical grade toluene was sodium dried and ready to use for the synthesis of MIPs.

7.3.3.2 Cross-linker – EGDMA

EGDMA was washed with 10% aqueous NaOH in brine and then dried over anhydrous magnesium sulphate and distilled under reduced pressure prior to use.

7.3.3.3 Monomer – MAA

MAA was dried over anhydrous magnesium sulphate and distilled under reduced pressure prior to use.

7.3.3.4 Initiator – AIBN

AIBN was dissolved in acetone at room temperature and then the solution was filtered and kept in the freezer to recystallize prior to use.

7.3.4 Synthesis of Ketamine MIPs and NIP

Ketamine MIPs were prepared using a bulk polymerization procedure following the method published by the Vlatakis group [196]. R/S-ketamine or S-ketamine (0.2758 g, 1.16 mmol), MAA (0.39 mL, 4.64 mmol) and EGDMA (4.5 mL, 2.32 mmol) were dissolved in toluene (6.7 mL) in a 25 mL, thick-walled glass kimax tube obtained from Sigma Aldrich. AIBN (0.0838 mg, 0.51 mmol) was then added and the solution was deoxygenated by bubbling oxygen-free nitrogen through the reaction mixture for 5 min while the flask was immersed in an ice bath. This procedure was to remove oxygen, which can retard the free radical reaction.

The kimax tube was sealed with the screw cap under a flow of nitrogen and placed on a Stovall flat-bed roller for 48 hours without introducing any speed or rolling (static). The temperature was kept at 60 °C for thermal polymerisation to occur. The template was extracted from the product MIPs with methanol/ acetic acid (9/1, v/v) for 24 h. A non-imprinted polymer (NIP) was prepared under the same conditions as the MIPs but in the absence of template.

The tubes were then smashed with a hammer and the MIP and NIP monoliths were separately crushed, mechanically ground using an automated grinding machine and wet-sieved using acetone. The retained particles were collected and allowed to sediment twice from acetone in order to separate the desired fractions from the finest particles. The end products obtained from the ground materials were in multiple sizes and were not all suitable for MISPE and LC analysis purposes. Only particulates of 25-38 µm size were collected for the purpose of the study. The products were weighed and dried overnight at 40 °C in a vacuum oven.

7.3.5 In-house Column Packing and Off-line Column Washing

Column Packing: The MIPs and NIP particles were packed into three liquid chromatography columns of dimensions 0.46 x 5.00 cm, with a frit size of 0.2 nm. Approximately 0.5 g polymers were packed in each column. An Altech Model 1666 Slurry Packer was used to pack the columns following procedures recommended by the manufacturer. Acetone was used as the slurry solvent. The

columns were packed at an air pressure of 500 psi over 20 minutes and about 100 ml acetone passed through the columns.

Column Washing: The purpose of column washing was to remove any ketamine which may bleed from the template. The columns were washed overnight off-line using a Gilson Model 303 pump with 95%:5% acetonitrile:acetic acid (v:v) with a flow rate of 0.5 mL/min at a pressure of 100 psi. The S-ketamine and R/S ketamine columns were washed simultaneously in tandem in the order S-ketamine column followed by R/S ketamine column to avoid any cross over.

7.3.6 High Performance Liquid Chromatography (HPLC) Analysis

In order to demonstrate the recognition properties of the MIP materials before being used for further analysis, they were evaluated using an HPLC system consisting of a Waters 1535 Binary pump, to which the columns were connected, a Waters 717 autosampler and a Waters 2487 dual wavelength absorbance detector. The software used was the Waters Breeze System.

The analysis was done using isocratic conditions. The mobile phase was 100% acetonitrile in Pump A with a flow rate of 0.5 ml/min. The sample volume was 10 µl and the detector wavelength was 220 nm. Sample injections on to the columns were carried out in the following order: pure acetone, 10 mM S-ketamine and 10 mM R/S-ketamine in the mobile phase. An imprinting factor was calculated by comparing the retention factors of the imprinted and non-imprinted columns. The peak areas were also compared.

The retention factor (k') was calculated as Equation 7-1

Equation 7-1 $$k' = t_r - t_0 / t_0$$

Where t_0 is the retention time of the void marker (acetone) and t_r is the retention time of the target analyte.

The imprinting factor (IF) was calculated using the Equation 7-2:

Equation 7-2 $$IF = k'MIP / k' NIP$$

Where *k' MIP* is the retention factor on the MIP column and *k' NIP* was the retention factor on the NIP column.

7.3.7 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) imaging was carried out at the SEM unit, Chemistry Department, University of Glasgow, using a Joel 6400 instrument. Very small samples of the polymer powders were sputter-coated with gold prior to SEM. SEM was used to image the size and shape and also the porosity of the polymer particles but not to measure the degree of polymer imprinting [178].

7.4 Results and Discussion

This study used a non-covalent approach for making MIPs where complex formation is the result of non-covalent or self assembly coordination interactions in the reaction mixture. These methods are more flexible in the range of chemical functionalities which can be targeted and thus the range of template that can be used [197]. Two MIPs, R/S- and S-ketamine, and a corresponding non-imprinted polymer (NIP) were made as shown in Table 7-1 using the most common method for MIP preparation, bulk polymerization.

Table 7-1 MIPS and NIP prepared using monolith polymerization

Polymer	Template/ monomer cross-linker	Template (mg)	Monomer MAA (mmol)	Cross-linker EGDMA (mmol)	Porogen Toluene (mL)	Initiator AIBN (mmol)
(NIP)	0:4:20	0	4.64	2.32	6.67	0.51
S-MIP	1:4:20	0.26	4.64	2.32	6.67	0.51
R/S-MIP	1:4:20	0.26	4.64	2.32	6.67	0.51

The materials were prepared on a 5 gram scale with the ratio of 1:4:20 for the template (ketamine), monomer (MAA) and cross-linker (EGDMA) in the presence of the porogen, toluene in appropriate volume. The polymerization reactions were carried out under thermal conditions at a temperature of 60ºC as shown in Fiqure 7-1. This procedure involves free radical polymerization and oxygen can retard the process. To ensure good polymerization occurred, oxygen was removed by displacement with an inert gas, nitrogen, for 5 min while immersed in an ice bath.

The ketamine MIPs prepared in this study were based on the most commonly used monomer, cross-linker, initiator and porogen with the intention that the template ketamine has a high affinity and rebinding selectivity to the polymer. Furthermore, the template had to withstand elevated temperature for thermal polymerization at 60ºC and this was studied before selecting ketamine as the template. The MIP preparations in this study were easy and not cost- and time-consuming and the materials obtained were stable and could be stored at room temperature, in contrast to natural receptors such as antibodies and enzymes.

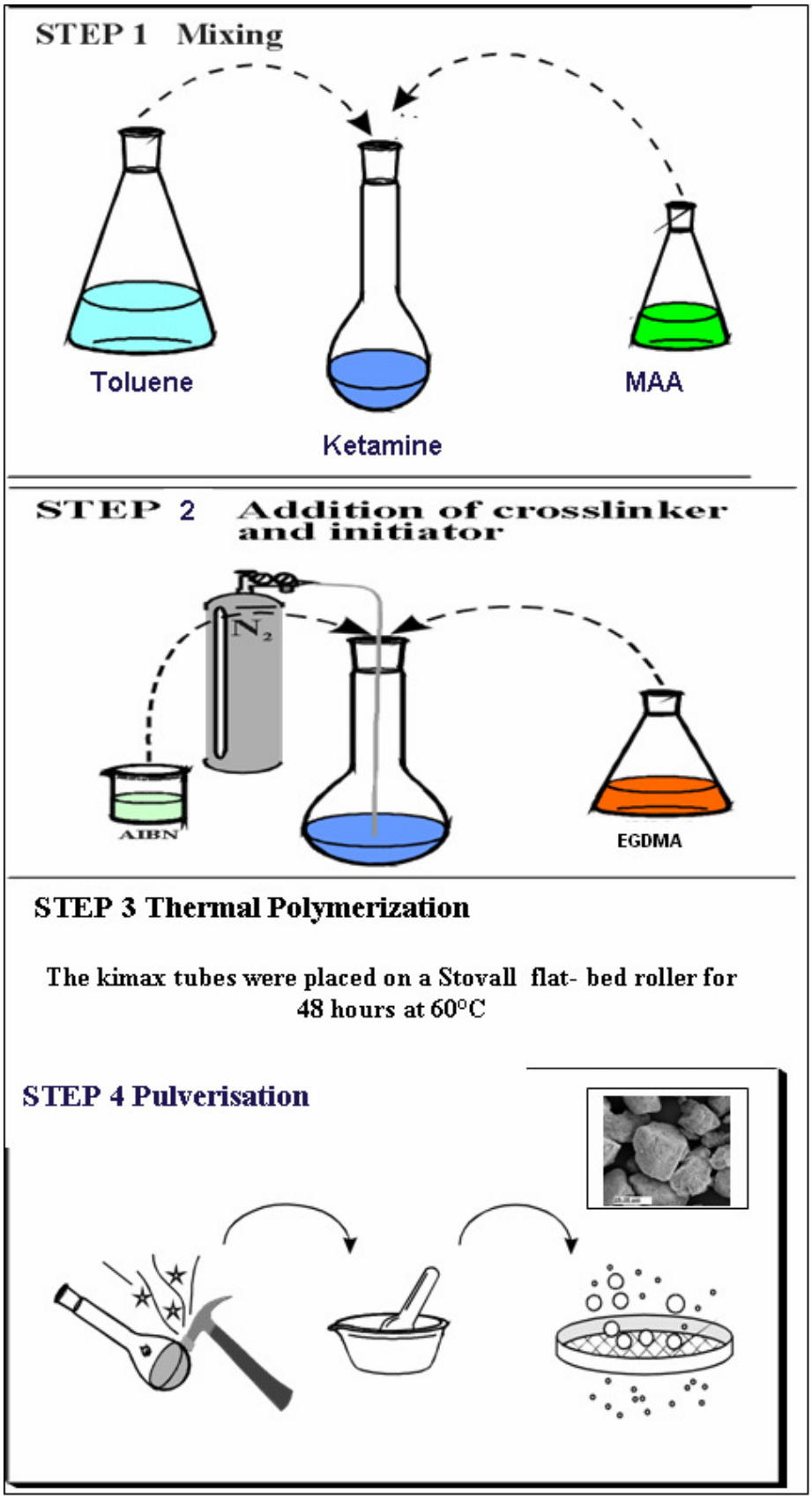


Figure 7-1 Schematic diagram of the MIP preparation process

As known from previous studies, the materials resulting from bulk polymerization were irregular with respect to particle size and shape. The MIPs and NIP obtained in this study correlated with the previous findings as shown in Figure 7-2. For the purpose of this study, only 25-38 µm particles were selected because particle sizes under 25 µm were not suitable for MISPE due to the difficulty of obtaining suitable frits for SPE columns.

The SEM (x 1000) photograph showed that the particle sizes were in the range 25 -38 µm as desired and so were suitable for use in the next study. The irregular size and shape of the particles produced by this method may result in poor chromatographic performance and can increase column back pressure. However, there have been many successful studies using MIP materials produced by bulk polymerization.

For example, Caro et al. successfully determined enrofloxacin and ciprofloxacin, which are used to promote animal growth and may endanger human health, below the maximum residue limits established by the European Union using two SPE steps, including MISPE [198]. Another example was a successful study of the synthesis and evaluation of molecularly imprinted polymers for extracting hydrolysis products of organophosphate flame retardants by Moller et al. which used a structural analogue, ditolyl phosphate, as template, instead of the target compound triphenyl phosphate [199].

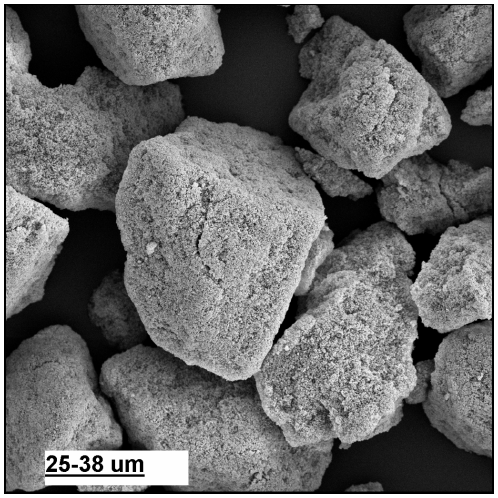


Figure 7-2 SEM micrograph (x 1000) of an S-ketamine MIP obtained from bulk polymerization process).

Chromatographic evaluation of ketamine-imprinted polymers was carried out using HPLC with a UV detector. The purpose of this evaluation was not to determine the detection limit of ketamine but to measure the imprinting factor of the MIPs, both racemic and S-enantiomer. For that reason, the instrument conditions were not optimised.

The detector wavelength was set at 220 nm based on a previous study of ketamine detection by HPLC [107]. Measurements were carried out in duplicate and the results indicated that the columns were reproducible, as shown in Table 7-2.

Table 7-2 Retention Time and Peak Area data of HPLC-UV for NIP, S-MIP and R/S-MIP ketamine columns

Column	Injections (10 µl)	Retention Time (min)	Peak Area (uV*sec) & Relative Peak Area (in bracket)
NIP	Acetone	1.5	5173188 (1.0))
	10 mM S-ketamine	2.4	12182852 (2.35)
	10 mM R/S ketamine	2.5	12930074 (2.49)
	Acetone	1.57	5177303 (1.0)
	10 mM S-ketamine	2.4	13134462 (2.53)
	10 mM R/S ketamine	2.4	13633078 (2.63)
S-MIP	Acetone	1.6	5467569 (1.0)
	10 mM S-ketamine	7.3	56548754 (10.34)
	10 mM R/S-ketamine	5.6	54877542 (10.03)
	Acetone	1.6	5567438 (1.01)
	10 mM S-ketamine	7.3	56548754 (10.34)
	10 mM R/S ketamine	5.7	55098623 (10.07)
R/S-MIP	Acetone	1.6	5440285 (1.0)
	10 mM S-ketamine	5.9	56548754 (10.39)
	10 mM R/S ketamine	5.7	53253862 (9.78)
	Acetone	1.6	5503451(1.01)
	10 mM S-ketamine	5.9	56047906 (10.30)
	10 mM R/S ketamine	5.7	53256879 (9.79)

Acetone was used as the void marker due to its lower affinity towards the polymers and therefore it had a shorter retention time than ketamine. Acetone as the void marker can be used as indicator to assess the column packing. The good, symmetrical peak shape of acetone on the NIP and MIP columns indicated that the columns had been well packed.

HPLC evaluation of the MIP columns was done by comparing the affinity of the MIP columns towards the target analytes with the NIP column which was similarly synthesised and packed but without the presence of the template. The retention factors (k') and the imprinting factors (IF) for the MIP and NIP columns

were calculated according to Equations 7.1 and 7.2 from the raw data in Table 7.2 using the average of the duplicates and the results were tabulated as shown in Table 7-3. The imprinting factor was more than 1.0, indicating that the polymers have been imprinted.

Table 7-3 Retention Factors (k') and Imprinting Factors (IF) obtained during the chromatographic evaluation of the imprinted polymers

Compound/Column	Retention Factor (k')	Imprinting Factor(IF)
S ketamine on NIP	0.64	NA
R/S ketamine on NIP	0.64	NA
S-ketamine on S-MIP	3.45	5.39
R/S-ketamine on S-MIP	2.42	3.78
S-ketamine on R/S-MIP	2.61	4.07
R/S-ketamine on R/S-MIP	2.45	3.82

The results in Table 7.3 indicated that both racemic ketamine and enantiomerically pure S-ketamine (R/S- and S-ketamine) had been imprinted and enatioseparated on both R/S and S-ketamine MIP columns as shown by their imprinting factors of > 1.0. The results also indicated that the MIP columns (R/S- and S-ketamine) could recognise the target analytes (ketamine enantiomers) but each column (R/S and S-ketamine MIP) has different affinity and selectivity towards the enantiomers as shown by the value of their imprinting factors.

S-ketamine was retained more strongly on the S-MIP column compared to the R/S-MIP column, as indicated by the imprinting factors (5.39 > 4.07). From Figure 7.3 the S-MIP column can discriminate between the R/S and S-ketamine based on their retention times which showed that the S-ketamine was retained longer than the R/S-ketamine on the S-MIP column. The enantioseparation by

the S-MIP column therefore indicated the potential of chiral selectivity towards both ketamine enantiomers.

This finding was in agreement with the study by Hart et al., who demonstrated that MIPs for benzodiazepines were able to discriminate between enantiomers of the imprinted molecules and also a wide range of enantiomers of structurally related molecules [200]. The NIP captured some ketamine due to non-selective binding but the peak areas were lower than with the MIPs, indicating that the NIP had lower affinity and selectivity towards the ketamine enantiomers.

Both MIP columns (Figure 7.3 and Figure 7.4) gave broad peaks with extensive tailing for the ketamine enantiomers which indicated that imprinting had taken place and the analytes had high affinity of the columns.

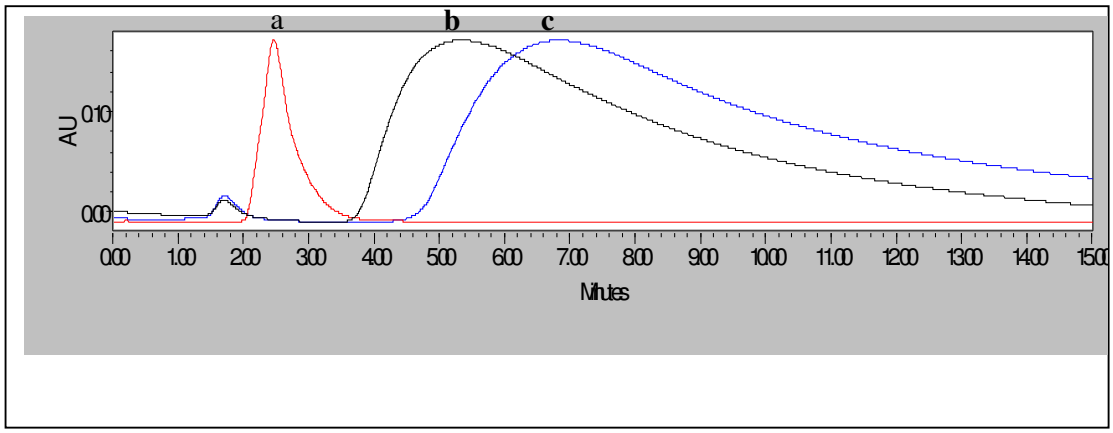


Figure 7-3 Separation of enantiomers on the S-ketamine MIP column using HPLC-UV
a = acetone, b = R/S-ketamine and c =S-ketamine

The imprinting factors of S-ketamine and R/S-ketamine on the R/S-MIP column were quite similar due to the close retention times of both ketamine enantiomers. Based on that, the R/S-ketamine MIP column did not show the enantioseparation characteristic for both ketamine enantiomers and the peaks of the R/S- and the S-ketamine overlapped as shown in Figure 7.4.

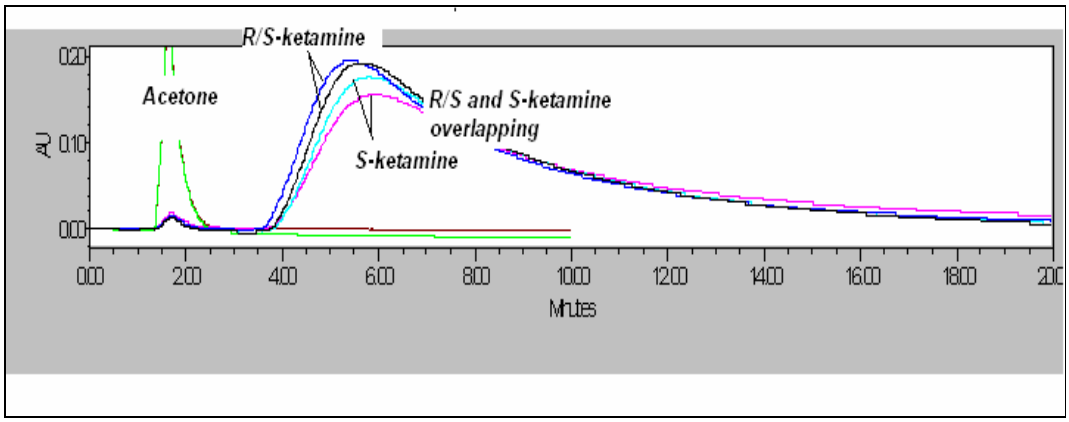


Figure 7-4 Separation on R/S-ketamine MIP column

Almost half of the drugs used are chiral, including ketamine. It is well known that both pharmacological effects and toxicity are related in most of cases to one enantiomer [201]. There can be differences in the activities of the enantiomers in which the pharmacologically inactive enantiomer can show unwanted side effects; in some cases antagonistic and even toxic effects are observed [202]. Therefore the chiral selectivity shown in this preliminary study is really very important because an HPLC column of this type could be used in forensic toxicology to distinguish the active isomer from the less active one.

The advantage of chiral phases based on MIPs is the high antibody-like selectivity as shown in this study. The limitation of this type of columns is the restricted range of applicability, since one special phase shows chiral recognition only for the same molecule used as template or very closely related compounds.

7.5 Conclusion

Molecularly imprinted polymers (MIP) for R/S- and S-ketamine have been prepared. Chromatographic evaluation using HPLC-UV demonstrated that the materials had been imprinted. The MIPs were ready and suitable to use for extracting ketamine (MISPE) from biological samples prior to further analysis using LC-MS. In addition to that, the S-ketamine MIP had the ability to discriminate between the racemate and pure S-enantiomer of ketamine and shows promise for use in chiral analysis of ketamine.

8 OPTIMISATION OF MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION (MISPE) OF KETAMINE AND APPLICATION TO HUMAN HAIR SAMPLES

8.1 Introduction

The main goal in sample preparation is to obtain a clean extract with a high recovery of analyte of interest. There is a general requirement in forensic toxicology to increase the sensitivity of analytical methods in order to determine low concentrations of drugs in complex biological matrices such as urine, blood and hair which may contain endogenous components that can interfere with the quantification of the analytes [170]. Molecularly imprinted polymers have gained an important role in recent years as SPE sorbents for the pre-concentration and extraction of analytes of interest in biological samples [192,203,154].

The aim of this study was to optimise and evaluate the use of MISPE columns prepared in-house for ketamine extraction as well as to evaluate the use of MISPE in forensic toxicology in future as an alternative to conventional extraction methods. The principal intended application was hair analysis.

8.2 Introduction to Hair Analysis

The first toxicology testing of hair was carried out by the Baumgartner group 30 years ago, using radioimmunoassay for determining opiate abuse histories [204]. Since then, hair has been used as an alternative to body fluids such as blood, urine and tissues which are only able to provide short term information of drug exposure, within 1-5 days prior to sample collection [205]. There are a few advantages of using hair analysis such as non-invasive sample collection, reducing risk for sample adulteration, the ability to reveal chronic drug use and the application of sectional analysis to estimate the approximate time of drug exposure and, most importantly to gain long-term information on the magnitude of drug use. The limitations of hair sample analysis include limited sample weight and low drug concentrations.

The life span of a single hair varies from about 4 months to 4 years. Human hair grows at approximately 1 to 1.5 cm per month and the rate is dependent on factors including anatomical location, and the race, sex and age of the person [206].

A single strand of hair consists of a root and a shaft. The hair bulb and follicle are part of the root, while the shaft consists of medulla, cortex and cuticle. After ingestion, drugs will circulate in the bloodstream including the blood vessels of the hair bulb. They can diffuse out of the blood vessels and become entrapped in the core of the hair shaft as it grows out from the hair follicle. Therefore, hair samples are suitable for determining drug abuse histories.

Hair analysis cannot be used as the sole evidence in a court of law, but only to support other evidence of drug use, due to unsolved controversies such as passive contamination, mechanism of drug incorporation, effectiveness of wash procedures and bias of different cosmetic hair treatments, as proposed by the Society of Forensic Toxicologist (SOFT) in 1992 [15].

There are a few published reports of ketamine hair testing as the individual drug or in combination with other drugs using GC-MS/MS [207,208]. The current study pioneered a radical approach by using MIP as sorbent for the solid phase extraction in tandem with LC-MS/MS analysis. This approach has never before been reported for the determination of ketamine and metabolites in the forensic toxicology field.

8.3 LC-MS/MS Conditions

LC analysis was carried out using (A) 3 mM aqueous ammonium formate plus 0.001% formic acid and (B) acetonitrile as mobile phase in a gradient elution program as shown in Table 8-1. Chromatographic separation was performed using a Synergi Hydro RP column (150 mm x 2.0 mm ID, 4 µm particle size) from Phenomenex, which was equipped with a guard column (4.0 mm x 2.0 mm, 4 µm) containing the same stationary phase. Ionization of analytes was performed using electrospray ionisation (ESI) in the positive ion mode and data was collected in the Selective Reaction Monitoring (SRM) mode and processed using Xcalibur Software version 1.3 also from Thermo Finnigan.

The LC-MS-MS method was based on a published method for the analysis of multiple drugs in hair, including amphetamines, benzodiazepines, cocaine and opiates [209]. Details of the LC-MS/MS conditions are explained in Chapter 5. In the present analysis the running time was shortened to 25 min and the flow rate was increased from 250 µl/min to 300 µl/min.

Table 8-1 LC-MS/MS conditions

Time (min)	3 mM ammonium formate + 0.001% formic acid (A %)	Acetonitrile (B %)
0	75	25
6	75	25
15	20	80
17	10	90
18	10	90
18.5	75	25
25	75	25

8.4 MISPE Method Development and Optimisation

Method development, optimisation and evaluation of ketamine MISPE included the column packing procedure, template removal, choice of conditioning, washing and elution solvents, binding capacity studies and cross-reactivity to the metabolites and other drugs.

8.4.1 MISPE Column Preparation

To prepare the MISPE columns, 20 mg ± 0.5 of *(±)*-ketamine MIP and NIP were weighed into culture tubes. Polyethylene frits with a pore size of 20 µm were inserted into empty 1 ml polypropylene SPE cartridges. The cartridges were connected to a vacuum manifold. The MIP and NIP in the culture tubes were slurried with 3.0 mL acetonitrile and the slurry was transferred to the empty SPE cartridges and sedimented with application of vacuum, in order to ensure the particles were uniformly packed into the cartridges [210]. The cartridges were

subjected to vacuum for 30 s before insertion of second frit on top of the sorbent beds. The cartridges were labelled for identification purposes. In total, 27 MIP columns and 30 NIP columns were prepared. Figure 8-1 shows examples of a *(±)*-ketamine MIP column, NIP column and empty column.

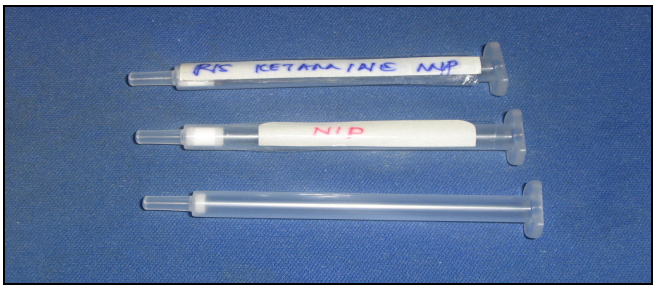


Figure 8-1 Examples of *(±)*-ketamine MIP, NIP column and empty columns.

8.4.2 Template Removal

This test was carried out because of a published study [211] which reported that there was an amount of template still remaining in the MIP even after extensive washing with acid and organic solvent using a Soxhlet extraction. The template remaining in the MIP was assessed by running 1 mL of acetonitrile with 5 % acetic acid (v/v) through the prepared columns (n=3). The eluates were collected and evaporated under nitrogen and then reconstituted with the mobile phase prior to the LC-MS/MS analysis. A calibration curve of 0-200 ng spiked unextracted ketamine was constructed for the determination of remaining template. In this study, approximately 32 ng ketamine was eluted from 20 mg polymer; it was necessary to remove the retained ketamine before the polymer could be used in the MISPE protocol.

In order to remove the template totally, the MIP cartridge was treated several times in series with different solvents as follows: 1 ml (90:10 v/v) methanol: acetic acid, 1 mL acetonitrile, 1 mL chloroform and 1 mL of 30 % acetic acid in acetonitrile. This approach to template removal has been successfully used in a previous study [212] but using different solvents for the treatment. This approach helped to release the template by promoting polymer swelling and disruption of template/polymer interactions. After each treatment cycle, the 30 % acetic acid/acetonitrile fraction was collected, evaporated and reconstituted with the

LC-MS mobile phase prior to analysis. Between treatments, the MIP sorbent was allowed to dry.

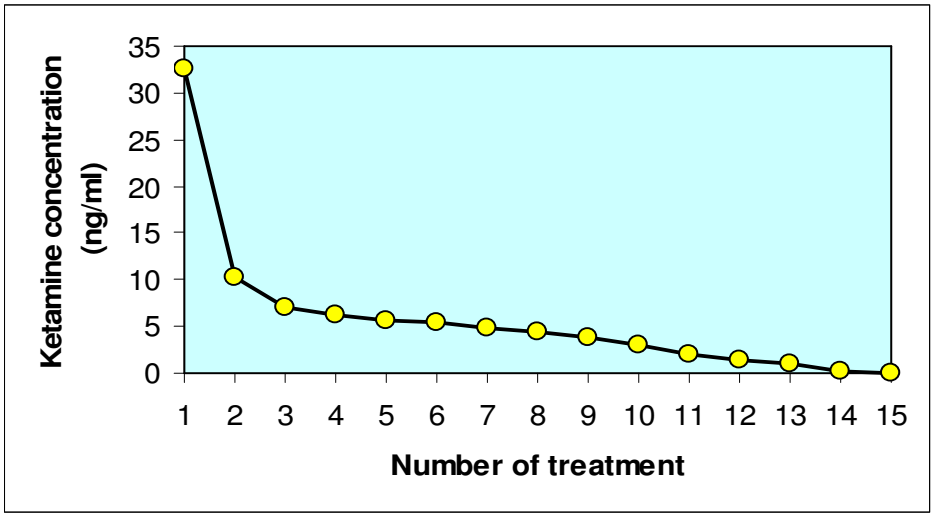


Figure 8-2 Reduction of template bleeding over 15 washes

The results showed that there is no more observable template bleeding from the polymer when treated more than 14 times with washing solution cycles (Figure 8-2). Therefore, all the cartridges were treated with these solutions prior to being used in the extraction procedure. We experienced difficulty in removing the ketamine template, most probably due to a strong interaction between the ketamine template and the polymer, and also the template could be trapped within the polymer matrix in non-specific locations, like a clathrate complex, as suggested by the chromatographic evaluation of the MIP which gave an imprinting factor of 3.78 for (±)-ketamine on the (±)-ketamine column.

8.4.3 Evaluating the Elution Solvent

A stepwise gradient of 5 to 50 % acetic acid acetic acid in acetonitrile was used for washing the MIP columns (n=3) to select the optimised elution solvent for the extraction steps. Columns were spiked with 250 ng of ketamine standard and washed, beginning with the lowest to the highest concentrations of acetic acid in acetonitrile. The last trace of spiked ketamine was eluted with 30 % of acetic acid in acetonitrile (v/v), as shown in Figure 8-3. This percentage of acetic acid was therefore used as the elution solvent for the extraction.

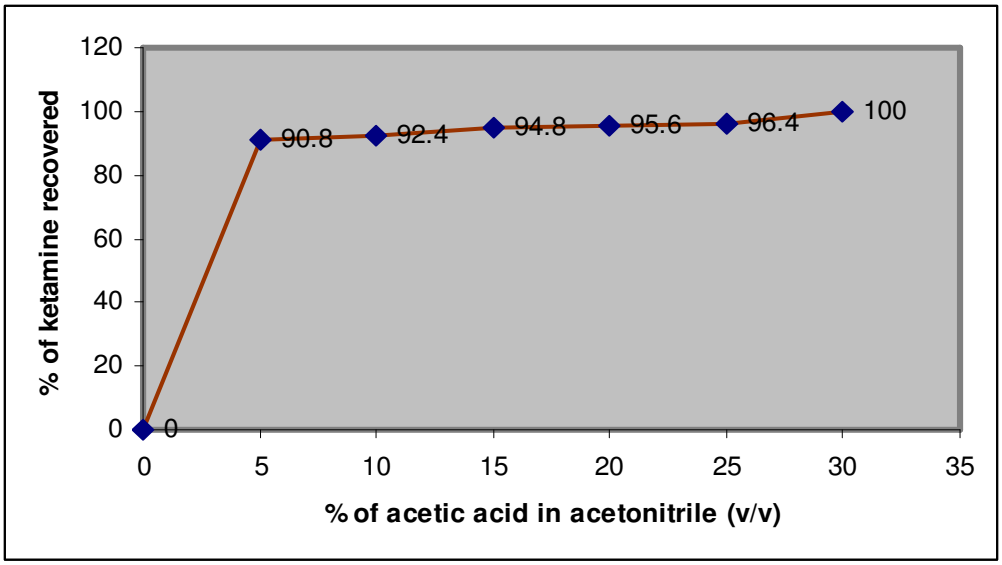


Figure 8-3 Effect of eluting solvent composition on recover of ketamine from the MIP.

8.4.4 Effects of conditioning and loading solvent on ketamine binding to MIP and NIP

This test was performed to assess the effect of different loading solvents in the recognition step. For those cases where recognition is driven by hydrogen bonding, the interaction forces between the analyte and the polymer are diminished when increasingly polar solvents are applied. To determine the effect of solvent on the ketamine MIP, the imprinted and non-imprinted polymers were conditioned with several alternative solvents, namely 1 mL chloroform, dichloromethane, toluene or acetonitrile prior to loading 50 ng ketamine in 1 ml of the same solvent. Vacuum was not applied to give the solvent and the MIP time to equilibrate and the solvent dripped through the MIP cartridges under gravity flow.

The cartridges were then washed with 0.5 ml of the solvent used in the loading step before eluting with 1.0 ml 30 % (v/v) acetic acid in acetonitrile. The fractions collected after the loading (FL), washing (FW) and elution (FE) steps were evaporated using a stream of nitrogen gas and reconstituted in 60 µl of the LC-MS/MS mobile phase, 20 µl of which was injected for analysis. The percentage of ketamine bound to the polymer was calculated using Equation 8-1,

which is based on the assumption that 100 % of the ketamine loaded was eluted at the end of the extraction.

Equation 8-1 % bound = (peak area for FE/peak area of (FL + FW + FE)) x 100

The percentages of ketamine which bound to the polymers using four different solvents in the conditioning and loading protocol are shown in Table 8-2. The percentages of ketamine bound to the MIP were much higher than to the NIP, demonstrating the imprinting of the polymer. This data may enable the optimal solvent for use in further experiments in this subsequent work to be identified.

According to the theory of molecular imprinting, an MIP tends to exhibit optimal molecular recognition when in the presence of toluene, the best porogen to use during polymerization. However, the percentage of ketamine bound was higher when using chloroform, suggesting that stronger binding occurs in the more polar solvent. In other words, the solvent with the higher dipole moment (chloroform) is the more effective solvent for rebinding in this study.

Table 8-2 Effect of solvent on ketamine binding to the MIP and NIP

Solvent	Dipole Moment Debyes (D)	% bound	
		MIP	NIP
Toluene	0.38	18	4
Chloroform	1.08	66	1
Dichloromethane	1.14	57	10
Acetonitrile	3.44	35	3

When ketamine was reintroduced to the ketamine MIP sorbent, the binding was best in chloroform, followed by dichloromethane, acetonitrile and toluene as shown in Figure 8-4. It was noticed that some ketamine was lost in both the loading and washing steps with all solvents. This may be explained by slow kinetics in the interaction between the polymer and the analytes. These experiments (n=3) demonstrated that the cartridges worked when using organic solvents and gave rise to various recovery percentages depending on the

selection of solvent. Next, the cartridges were used for extraction of ketamine extraction from aqueous media.

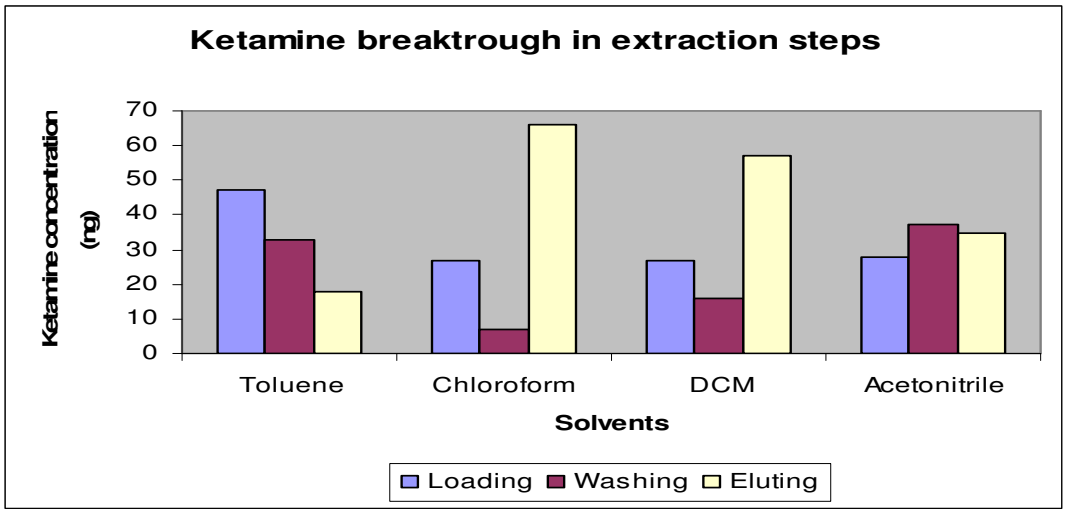


Figure 8-4 Effects of solvent on ketamine MIP binding.

From the results shown in Figure 8-4, it was also found that chloroform was suitable for use as the washing solvent to deliver cleaner extracts with the highest ketamine recovery during the extraction process. The breakthrough of ketamine in MIP and NIP using different solvents in extraction steps is shown in Figure 8-5.

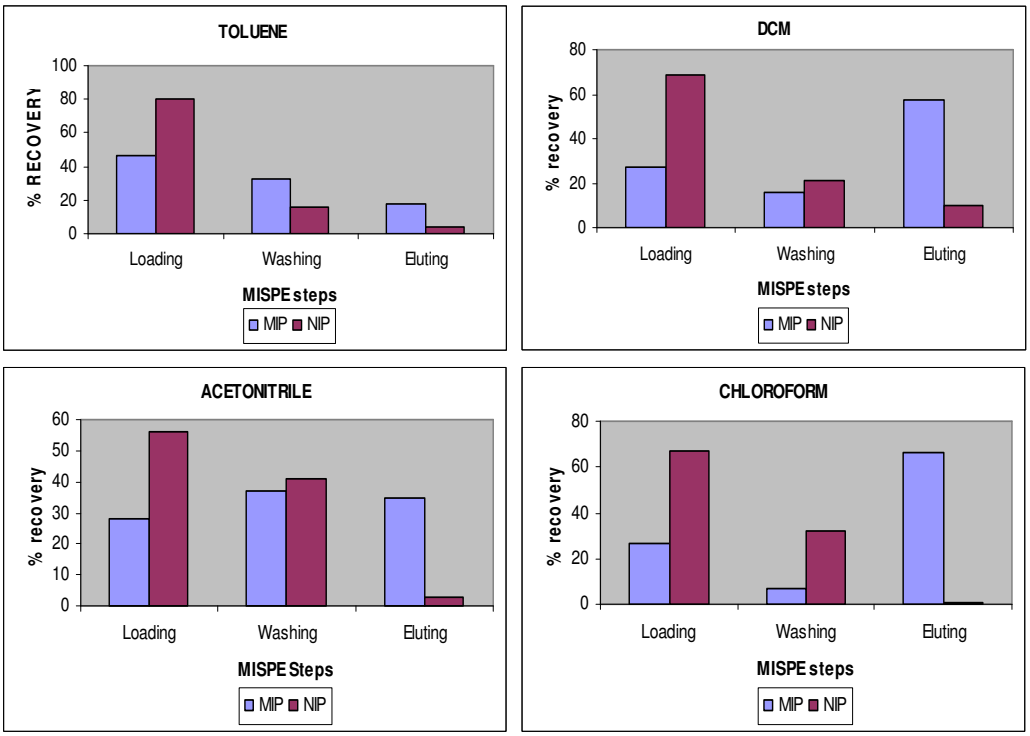


Figure 8-5 Comparison of breakthrough of ketamine using MIP and NIP columns.

8.4.5 Problem of Using Chloroform

To test the method in aqueous samples, 0.1 to 100 ng of ketamine was spiked in 1 mL of phosphate buffer 0.1 M pH 5.0. The cartridge was preconditioned with 3 mL of acetonitrile followed by 1 mL of the phosphate buffer. The MIP column was conditioned with acetonitrile followed by phosphate buffer for activating the MIP column because the hair was pre-extracted in phosphate buffer before loaded into the MIP cartriges. In this aqueous sample, chloroform was not used even though the solvent indicated to be the best conditioning solvent in organic environment. Each sample was loaded on a cartridge which was then washed with 1 mL chloroform. The analytes retained in the cartridge were eluted with 1 mL 30 % (v/v) acetic acid in acetonitrile. 100 ng of internal standard was then added after the extraction.

The results showed that there was no linear calibration of MIP extraction when using chloroform as the wash solvent. It was seen that there were two layers of solvent when chloroform was used in the washing step due to immiscibility of the buffer and chloroform. This may be explained by elution of trapped

template from the polymer following the sudden change of solvent from phosphate buffer (polar) to chloroform (non-polar) which altered the ratio of ketamine to internal standard and affected the linearity of the calibration curve.

Subsequently chloroform was not used in the washing step but was replaced with an alternative, more polar, solvent such as pure acetonitrile or 1 % acetic acid in acetonitrile to avoid template bleeding due to polymer shock.

8.4.6 Recovery of Ketamine using MIP and NIP, with 0.1M Phosphate Buffer pH 5 as the loading solvent

In this experiment, 50 ng of ketamine was spiked in 1 mL of 0.1M phosphate buffer pH 5.0 (n=3). MIP and NIP cartridges were preconditioned with 3 mL of acetonitrile followed by 1 mL of the phosphate buffer. Each sample was passed through a cartridge and then eluted with 1 mL 30 % (v/v) acetic acid in acetonitrile. The recoveries of ketamine from the MIP and NIP are shown in Figure 8-6.

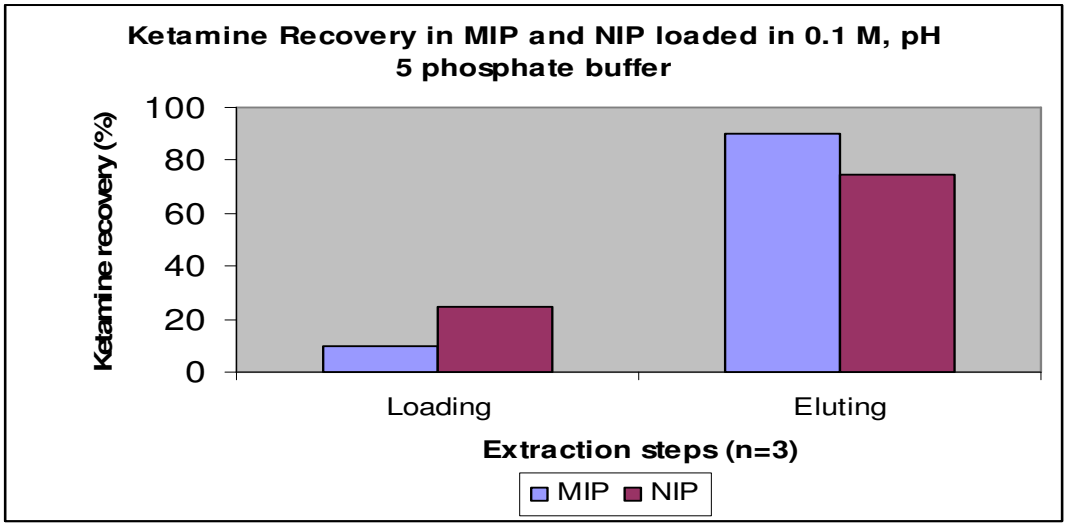


Figure 8-6 Recovery of MIP and NIP without washing step

Analytes present in samples are retained on both MIP and NIP under aqueous loading conditions due to non-specific hydrophobic interactions [155] which explains the data shown in Figure 8-6. In this regard, a washing step of 0.5 ml 1 % acetic acid in acetonitrile was added to the SPE protocol to "switch on" the

molecularly selective retention mechanism of the MIP. The results are shown in Figure 8-7.

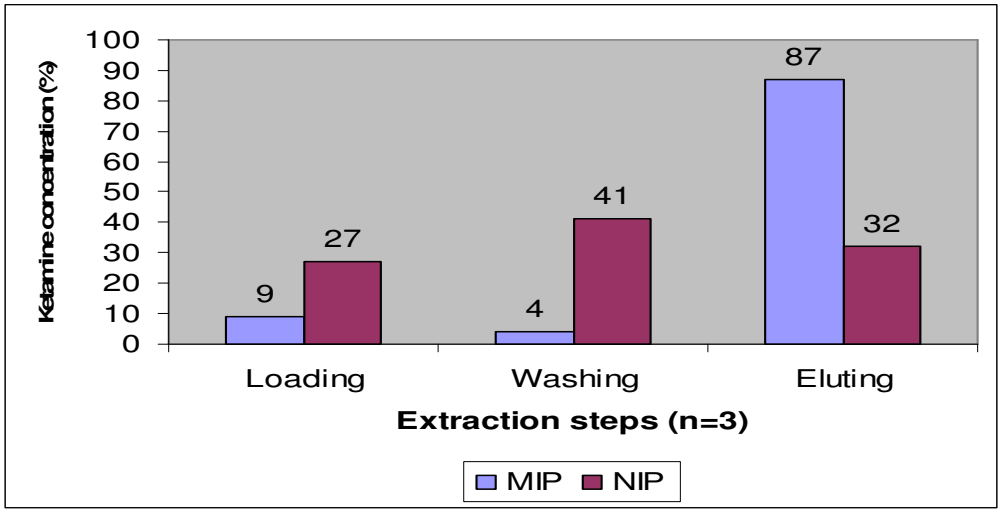


Figure 8-7 Recoveries of MIP and NIP with washing step

This experiment showed that the selectivity of an MIP for an analyte depends on the careful choice of the extraction procedures as demonstrated in other studies, for example by the Baggiani group [213] for the clean up of phenytoin from plasma samples. The washing step helped to produce a cleaner extract with an acceptable analyte recovery. The final optimised MISPE method is shown in Table 8-3.

Table 8-3 Optimised MISPE protocol for extraction of ketamine from aqueous samples

Extraction Steps	Solvents
Conditioning	3.0 mL acetonitrile; 1.0 mL 0.1M pH 5.0 phosphate buffer
Loading	ketamine in 1.0 mL 0.1M pH 5.0 phosphate buffer
Washing	0.5 mL of 1 % acetic acid in acetonitrile
Eluting	1.0 mL 30% acetic acid in acetonitrile

8.4.7 Cross-reactivity of Ketamine MIP to Morphine, PCP and Tiletamine

It has been postulated that the selective molecular recognition within the molecularly imprinted cavity can be attributed not only to cooperative binding of pre-organized functional groups but also to the shape selectivity that is complementary to the template [214]. In this study morphine, PCP and tiletamine were selected to investigate the role of shape selectivity of the (±)-ketamine MIP. 50 ng morphine, PCP and tiletamine were subjected to the same extraction procedure as used for ketamine in triplicate. The molecular structures of morphine, PCP and tiletamine are shown in Figure 8-8.

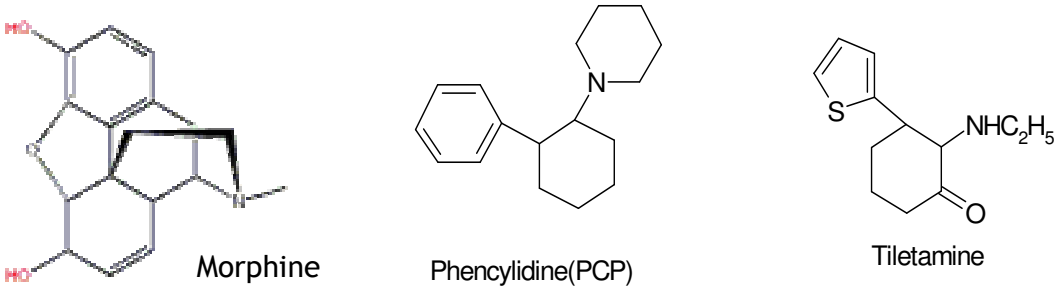


Figure 8-8 Molecular structures of morphine, PCP and tiletamine.

The eluents were collected from the extraction and two unextracted samples containing the same amounts of morphine, PCP and tiletamine were blown down and reconstituted in the mobile phase prior to the LC-MS/MS analysis. The percentage of morphine, PCP and tiletamine bound to the ketamine MIP was calculated using Equation 8-2.

Equation 8.2 **% bound = (peak area of extracted drugs/ peak area of unextracted drugs) x 100**

By using the same extraction protocol for the other drugs as for ketamine, the cross-selectivities of the MIP for morphine, PCP and tiletamine were found to be less than 5 %. The finding indicated that the analytes with different shape and different functional groups from those of the template ((±)-ketamine) has lower affinity towards the MIP, hence the MIP has decreased selectivity towards these

analytes. This finding was in agreement with the study by Spivak et al., which demonstrated that large analytes showed less selectivity when introduced to an MIP synthesised with a template that was smaller, when steric exclusion will reduce binding to the imprinted sites[214]. On the other hand, analytes smaller than the template have reduced contact with the polymer matrix that makes up the binding cavities, which results in lower Van Der Waals or hydrophobic interactions and reduced selectivity.

8.4.8 Binding Capacity of Ketamine MIP

Experiments for the determination of the ketamine binding capacity of the ketamine MIP cartridge were performed using the optimised procedure, with 0.1M phosphate buffer pH 5 as the solvent of choice. High concentrations of ketamine ranging from 500 to 3000 ng were dissolved in 1 mL 0.5M phosphate buffer pH 5 and extracted separately using different ketamine MIP cartridges (n=2). A mixture of 50 ng of each internal standard was added to the eluent after the extraction. The eluent was evaporated to dryness with a nitrogen stream and the residue was reconstituted with the initial mobile phase prior to analysis by LC-MS/MS.

A graph of peak area ratio of ketamine over the internal standard against the concentration of ketamine loaded was plotted as shown in Figure 8-9. This indicated that the level of ketamine binding was essentially constant when the MIP was loaded with 2500 ng of ketamine.

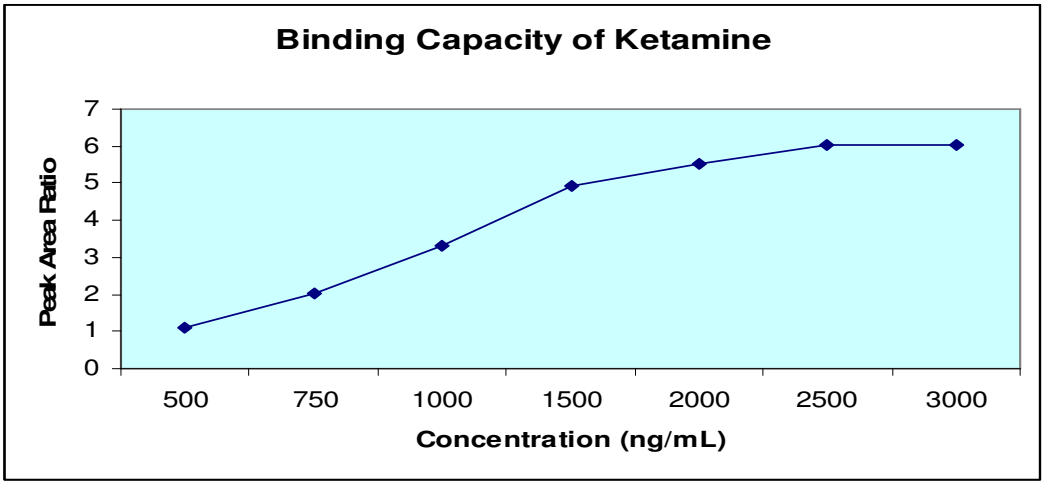


Figure 8-9 Ketamine binding capacity

From this result, a cartridge of 20 mg ketamine MIP sorbent bound 2500 ng ketamine, so the binding capacity of the imprinted polymer was 125 ng of ketamine per mg MIP. There is no previous literature about the binding capacity of an anti-ketamine MIP available for comparison. However, a binding capacity of 0.125 µg/mg polymer is lower than the average binding capacity of other MIPs recorded in the literature, which is 1-5 µg per mg polymer. Examples include a chlorpromazine MIP [215] with an absorption capacity of 10 µg per mg polymer and a 17β-estradiol MIP [216] with an absorption capacity of 3.6 µg per mg polymer. However, the binding capacity obtained in the anti-ketamine MIP was sufficient for the analytical work in this study due to the lower concentrations of drugs in hair.

8.5 Method Validation and Application to Hair Samples

8.5.1 Human Hair Samples

Human hair samples were provided by four females from a drug prevention centre in Malaysia. Samples were collected by a collaborating research unit at University of Science, Malaysia. Hair samples (approximately of 75 µm in diameter) were cut close to the scalp at the posterior vertex area. The hair samples were folded and put into aluminium foils prior to placement in paper envelopes. The hair samples were washed using hair shampoo, rinsed twice in water, sonicated for 10 minutes and dried. After drying, the hair samples were decontaminated and sonicated in 10 ml dichloromethane for 10 minutes at room temperature. The samples were then dried in a hot air oven at 45 ºC and then cut into very small pieces of less then 1 mm.

The ready cut samples were put into aluminium foils and envelopes and then mailed to Department of Forensic Medicine and Science, University of Glasgow. Upon receipt, the samples were stored in dark and dry conditions at room temperature. Samples Q, R, S, V and a mixed sample of Q, R, S and V are shown in Figure 8-10.

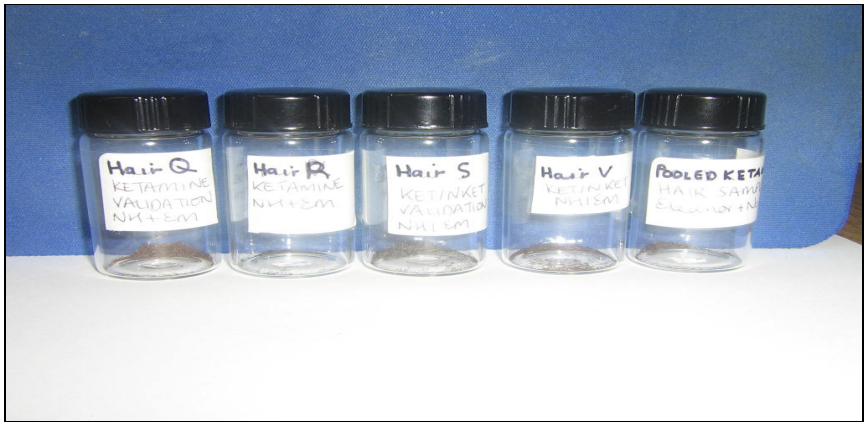


Figure 8-10 Hair Samples Q, R,S,V and a mixed sample of Q,R,S and V.

8.5.2 Preparation of Hair Samples

Blank hair samples were collected from volunteers in the laboratory. All samples were wrapped in aluminium foil and stored in a cupboard at room temperature. Hair samples were washed with 0.1% aqueous sodium dodecyl sulphate (SDS) and sonicated for 10 minutes. The hair samples were then rinsed and sonicated for 10 minutes, twice with deionised water and twice with dichloromethane, then left to dry in air. The dried hair samples were then weighed and approximately 10 mg samples were transferred to vials for the hair pre-extraction process.

In the hair pre-extraction process, 10 ± 0.1 mg samples were prepared by adding 1.5 mL 0.1 M phosphate buffer pH 5.0. Standards and internal standards ketamine-d4 and norketamine-d4 were added to the vials prior to the incubation. The vials were left to incubate for 18 hours at 45 °C. The hair extracts were cooled and transferred to clean vials.

8.5.3 Solid- Phase Extraction Using (±)-Ketamine MISPE Columns

MISPE was carried out using the in-house prepared ketamine imprinted SPE columns with the optimised procedure. Each cartridge was preconditioned with 3.0 mL acetonitrile followed by 0.1 M phosphate buffer pH 5.0. Each sample was passed through a cartridge and washed with 0.5 mL 1% acetic acid in acetonitrile

to remove interferences. The analytes retained in the cartridge were eluted with 1.0 mL 30% (v/v) acetic acid in acetonitrile.

8.5.4 Linearity, Precision and Stability Studies

Linearity was determined over the concentration range 0-100 ng per 10 mg spiked hair. 10 mg of blank decontaminated hair was spiked with 0, 2, 5, 10, 25, 50 and 100 ng of ketamine and norketamine standards and 100 ng of the internal standard mixture of each analytes. The hair samples were limited in terms of availability and 10 mg was choosing as the sample weight to suit the real hair sample analysis. The samples were then pre-extracted, extracted with MISPE and analyzed by LC-MS/MS. The calibration curves were constructed by plotting the peak area ratios of standards over internal standards against analyte concentration and were subjected to linear regression analysis.

The intra- and inter-day precision were determined by analysing the mixed samples, namely Pooled 1, Pooled 2 and Pooled 3, which were mixtures of the four individual hair samples. Because of the limited sample availability, only three samples were analysed in the same day (intra-day precision) and the experiment was carried out over three different days for inter-day precision values. Accuracies were not determined because the precision study did not have the target (true) concentration. The inter-day precision values were also used as a short stability study for the analytes in real human samples over a period of three weeks, as the pooled samples were analysed in three different weeks. The values were determined by comparing the results of analysis with those obtained when the calibration was done on the same day.

8.5.5 Matrix Effect Analysis

Six replicate extracts, each made with 10 mg of blank decontaminated hair, were spiked with 50 ng of both ketamine and norketamine (set a). Another six replicates were prepared containing the same concentration of ketamine and norketamine in 1 ml loading solvent, 0.1M pH 5.0 phosphate buffer (set b) prior to MISPE. 100 ng of internal standard mixture was added to each replicate in the two sets after the extraction. The percentage matrix effect was calculated according to Equation 8-3, where ‘a’ and ‘b’ are the peak area ratios of the

analyte to internal standard in human hair extract and phosphate buffer, respectively. Values were calculated to determine analyte suppression or enhancement due to matrix interference.

Percentage of matrix effect = a/b x 100% **Equation 8-3**

8.5.6 Limits of Detection (LOD) and Lower Limits of Quantification (LLOQ)

The LOD and LLOQ were determined for each drug using spiked hair. 10 mg of blank decontaminated hair was spiked with 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2 and 5 ng of ketamine and norketamine and 100 ng of the internal standard mixture of each ketamine and norketamine was added. The samples were then pre-extracted, extracted with MISPE and analyzed by LC-MS/MS. The LOD and LLOQ were calculated from the regression lines as the concentrations which gave signal-to-noise ratios of 3 and 10, respectively.

8.5.7 Recovery Study of Ketamine and Norketamine

(±)-Ketamine and (±)-norketamine were used to test the selectivity of the ketamine MIP. 50 ng samples of each drug were prepared in 1 mL 0.1 M phosphate buffer pH 5.0 (n=5). The samples were incubated and extracted to determine the recoveries of ketamine with the MIP. 100 ng of internal standard was then added after the extraction. The recovery was calculated by comparing peak area ratios obtained from extracted samples (quantitation ion/internal standard) to the peak area ratios of the same concentration of pure standards (quantitation ion/internal standard) in unextracted samples. The percent mean recovery and percent relative standard deviation (RSD) was calculated for each drug at each concentration.

8.5.8 Hair Case Samples

Four hair samples from Malaysia were tested in this study. Samples were cut and pre-washed by the collaborating research unit at the University of Science, Malaysia, as mentioned in Paragraph 8.5.1. 10 mg of blank decontaminated hair

was spiked with 0, 2, 5, 10, 25, 50 and 100 ng of ketamine and norketamine standards and 100 ng internal standards were added to each vial to generate a calibration curve. The samples were then incubated, extracted with MISPE and analyzed by LC-MS/MS according to the validated method.

8.6 Validation Results and Discussion

8.6.1 Linearity, Pecision and Stability Studies

8.6.1.1 Linearity

Ketamine and norketamine regression lines had $R^2 > 0.99$ over the concentration range 0-10 ng/mg. From the calibration graph shown in Figure 8-11, it can be observed that the response of the method (peak area ratios) for norketamine were much lower than ketamine for the same spiked concentration.

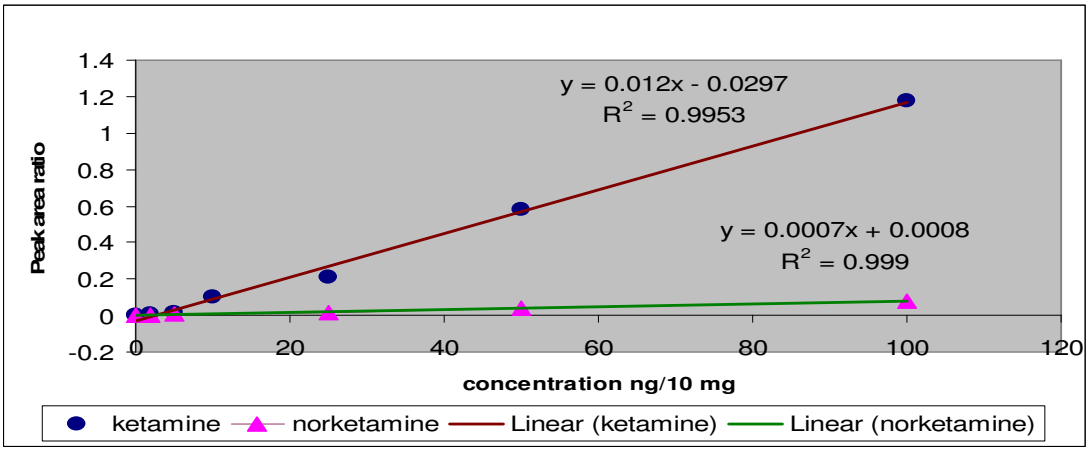


Figure 8-11 Calibration for ketamine and norketamine from 0-100 ng/10 mg hair

8.6.1.2 Intra-day and inter-day precision using authentic hair samples

The intra- and inter-day precision study was performed using unknown analyte concentrations from pooled samples to reflect the imprecision of real hair samples during analysis. The analyses were carried out using three replicates for intra-day precision and the same experiment was repeated for 3 separate days over a 3-week period for inter-day precision. Replicates were limited to three due to limited availability of sample. Ketamine and norketamine showed good

precision for both intra-day and inter-day, with low RSDs as depicted in Table 8-4.

Table 8-4 Intra-day and inter-day precision study using authentic pooled hair samples

Analyte	Intra-day (n=3) Pooled samples			Inter-day (n=3) x 3 different days Pooled samples		
	Mean	SD	R.S.D. (%)	Mean	SD	RSD (%)
Ketamine	2.81	0.07	2.37	2.63	0.20	7.90
Norketamine	0.88	0.07	0.9	0.87	0.01	7.5

8.6.1.3 Stability

A short stability study of ketamine and norketamine using the authentic pooled hair samples was carried over a period of three weeks. Samples (n=3) were washed and incubated and extracted following the optimised procedure. All samples were kept in the freezer (-20 °C) for up to three weeks prior to the next MISPE extraction and analysis by LC-MS/MS. The results of analysis of the second and third batches were compared with those obtained on the first day of analysis. There was no significant decrease for norketamine during a storage period of three weeks, when norketamine decreased by 1.7 %. However, ketamine decreased by 14.3 %. The results are shown in Table 8-5.

Table 8-5 Stability of ketamine and norketamine in freezer for three weeks

Pooled samples kept in freezer (n=3)		
Duration	**Ketamine concentration (ng/mg)**	**Percentage remaining**
Week 1	2.81	100.00 %
Week 2	2.70	96.4 %
Week 3	2.35	85.7 %
Duration	**Norketamine concentration (ng/mg)**	**Percentage remaining**
Week 1	0.880	100.00 %
Week 2	0.875	99.4 %
Week 3	0.865	98.3 %

8.6.2 Matrix Effect Study

Ion suppression is common in LC-MS analysis when analyzing trace levels of analyte(s) in complex matrixes such as hair samples. The matrix effect for ketamine showed minimal ion suppression of 6.8 % while norketamine showed a slight ion enhancement of 0.2 % for this particular set of samples. The matrix effect is often caused by the co-elution of matrix components with analyte(s) of interest and improved selectivity/sample clean-up during sample preparation is critical. Because the MIP used was targeted for ketamine, it was shown that the matrix effect was minimized when using MIP sorbents. In comparison, significant matrix effects were demonstrated in an unpublished study [217] using conventional SPE where the quantification ion of ketamine (m/z: 220) was suppressed by 47.3% and the quantification ion of norketamine (m/Z: 207) was enhanced by 62.2%.

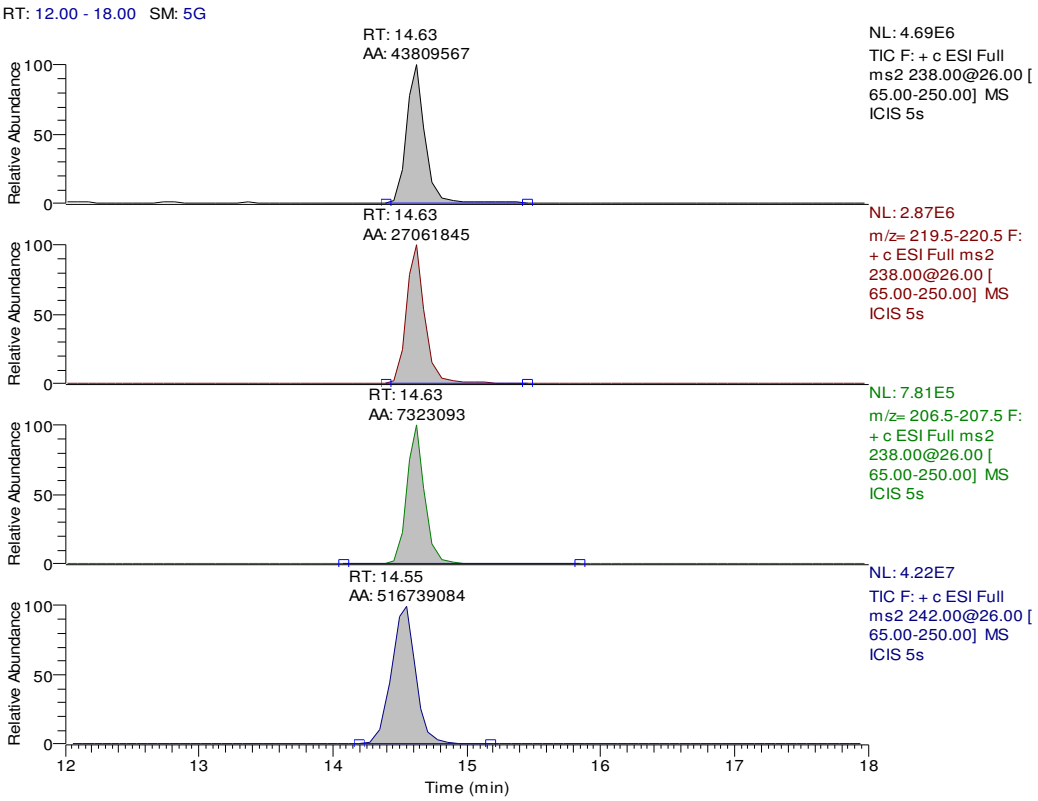
Table 8-6 Matrix effect using ketamine MIP columns

Analyte	Spiked concentration (ng)	Matrix Effect (n=6) (%) [RSD]
Ketamine	50	- 6.8 [12.3]
Norketamine	50	+ 0.2 [13.2]

8.6.3 LOD and LLOQ

The LOD and LLOQ values for ketamine were 0.1 and 0.4 ng/mg hair, respectively. The values were slightly higher for norketamine, with an LOD of 0.1 ng/mg and LLOQ of 0.5 ng/mg hair as shown in the chromatograms shown in Figure 8-12.

(A)Ketamine



(b) Norketamine

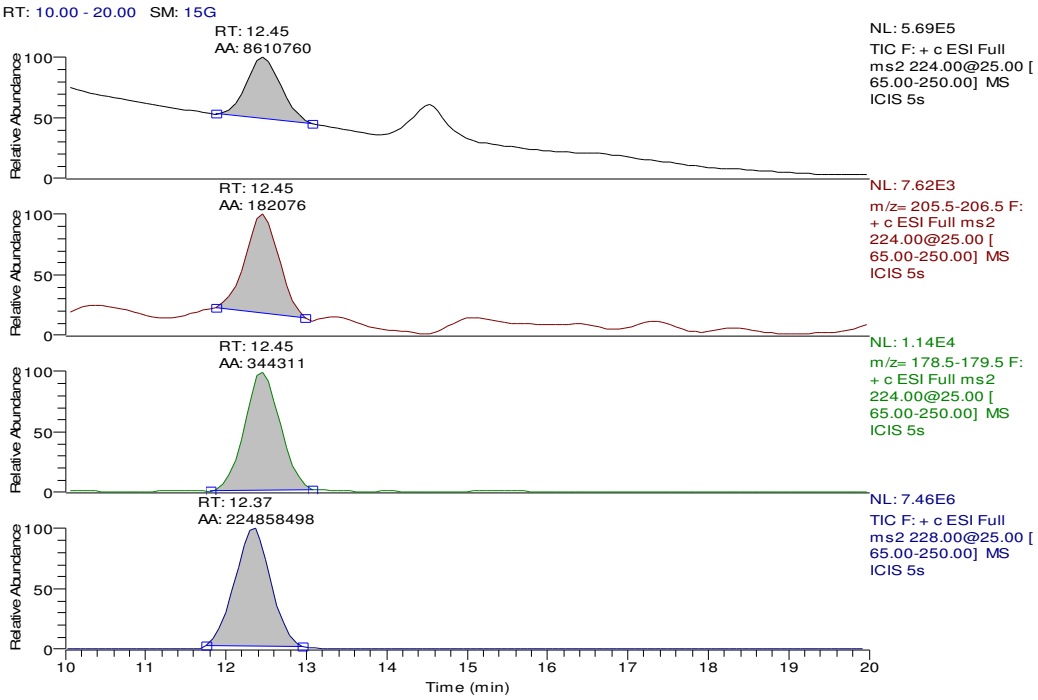


Figure 8-12 Quantification of (a) ketamine at 0.2 ng/mg and (b) norketamine at 0.5 ng/mg hair

The washing step prior to elution of analytes from an SPE cartridge helps to eliminate other interferences and can result in a cleaner chromatogram. As a consequence, the washing step added during the MISPE method resulted in cleaner extracts. Because extraction selectivity is significantly improved, lower background is observed, allowing the method to achieve lower detection limits as shown in Table 8-7.

Table 8-7 Analytical characteristics of LC–ESI-MS method for ketamine and norketamine

Compound	Linear range (ng mg^{-1} hair)	Regression line equation	Correlation coefficient (R^2)	LOD (ng/mg hair)	LLOQ (ng/mg hair)
Ketamine	0 - 10	Y = 0122x + 0.0297	0.9953	0.1	0.37
Norketamine	0 - 10	Y = 0.0007x + 0008	0.9990	0.1	0.47

By careful design of the polymerisation reaction and the resulting imprinting sites, the binding cavities can be engineered to offer multiple interaction points

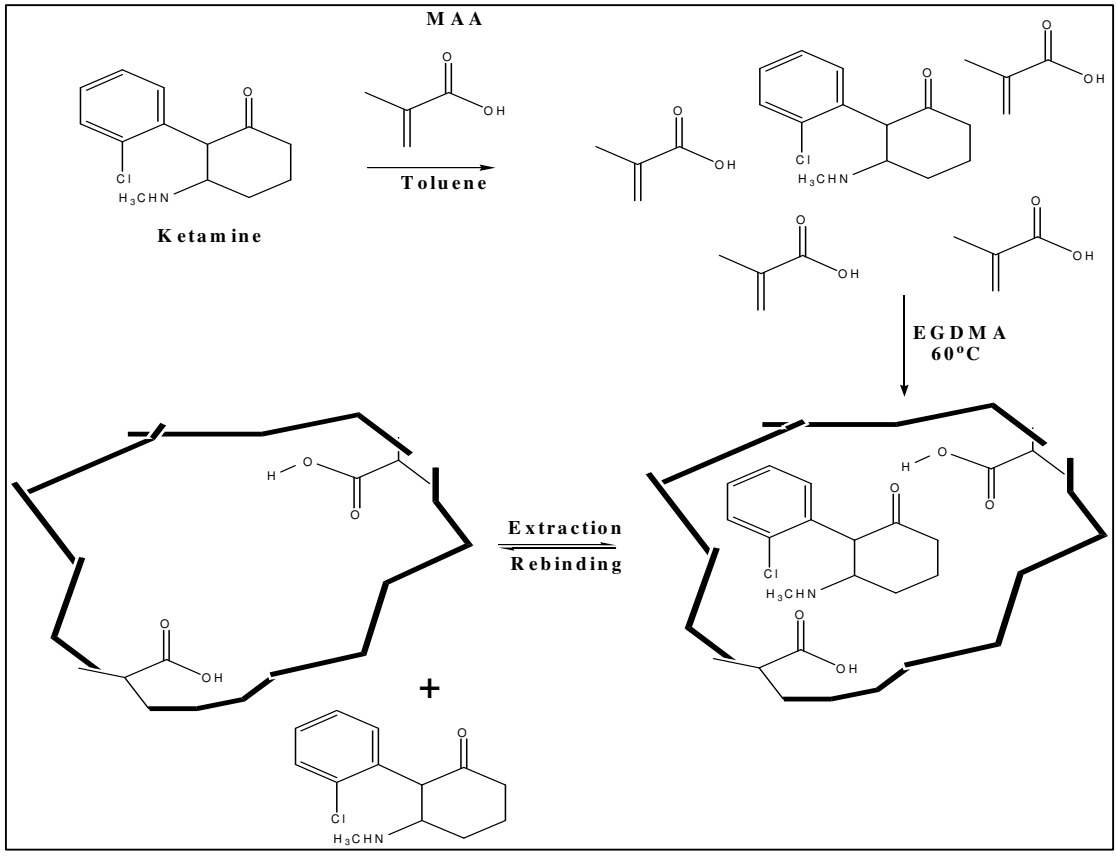


Figure 8-13 Created binding cavities for analytes rebinding

with the analyte(s) of interest. This leads to a stronger interaction between the sorbent and the analyte(s), and low limits of detection can be achieved.

8.6.4 Recoveries of Ketamine and Norketamine

The results showed that the MIP recovered a high percentage of ketamine and norketamine with acceptable RSD values. The results are tabulated in Table 8-8.

Table 8-8 Recoveries of ketamine and norketamine from hair.

Analyte	Spiked concentration (ng 10 mg^{-1})	MIP Recovery (%) (n=5)	RSD	NIP Recovery (%) (n=5)	RSD
Ketamine	50.00	86.12	9.34	31.54	18.94
Norketamine	50.00	88.37	5.04	27.82	22.67

The high recovery of ketamine from the MIP arises from the excellent molecular recognition of the template molecule imparted by the imprinting process. Lower recoveries were obtained when the NIP was used as the extraction sorbent, as expected, together with high percent RSD values. The lower RSD values may reflect the fact that the MIP is well-defined in respect to its binding compared to NIP.

8.6.5 Hair Sample Analysis

The four hair samples supplied from a drug abuse prevention centre in Malaysia were tested after the method was validated and ready for analysis of real samples. The hair samples were obtained from four females with a history of multiple drug use, including ketamine. The pre-cut and pre-washed hair samples were incubated extracted using MISPE and analyzed by LC-MS/MS according to the validated method. The results are shown in Table 8-9. In this study the application of the MISPE method successfully detected ketamine and norketamine for the chronic users; in future it may be useful for testing of single use applications such as date-rape cases.

Table 8-9 Hair sample weights, colours and concentrations of ketamine and norketamine

Sample Number	Weight MIP (mg)	Hair Colour	Ketamine concentration ng/mg	Norketamine concentration ng/mg
Q	19.96	Dark brown	4.4	present *
R	19.85	Black	5.7	1.2
S	20.02	Black	2.2	0.6
V	10.01	Light brown	0.2	present *

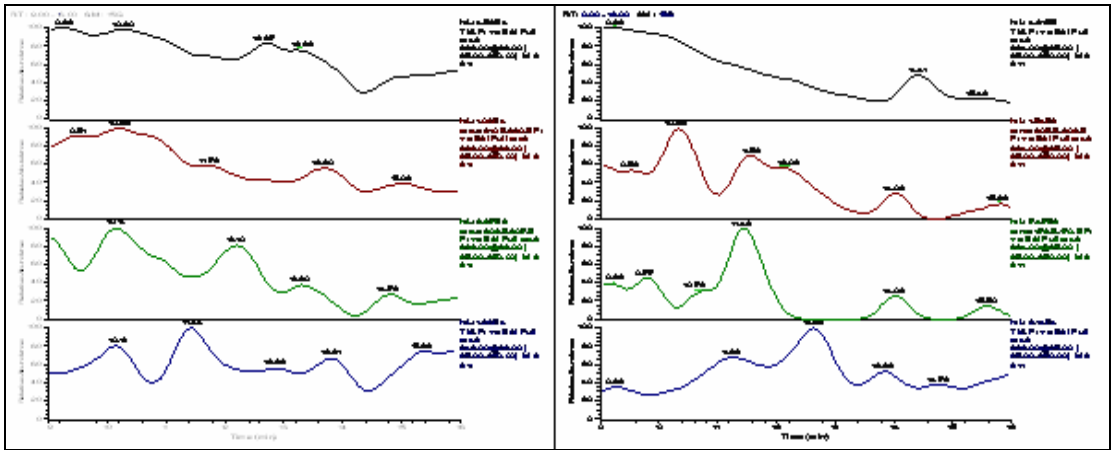
*** equal to or higher than the detection limit but lower than the quantification limit**

It is well known that drug deposition in hair is related to its lipophilicity. The change from a secondary amine in ketamine to a primary amine in norketamine increases the polarity and decreases the lipophilicity of norketamine compared to ketamine, therefore the concentration of ketamine in hair was higher than that of norketamine in this study. This concentration may also reflect the relative concentration of ketamine and norketamine in blood which was in agreement with the finding of Xiang et al. [101]. The ratio of norketamine to ketamine in hair was not consistent.

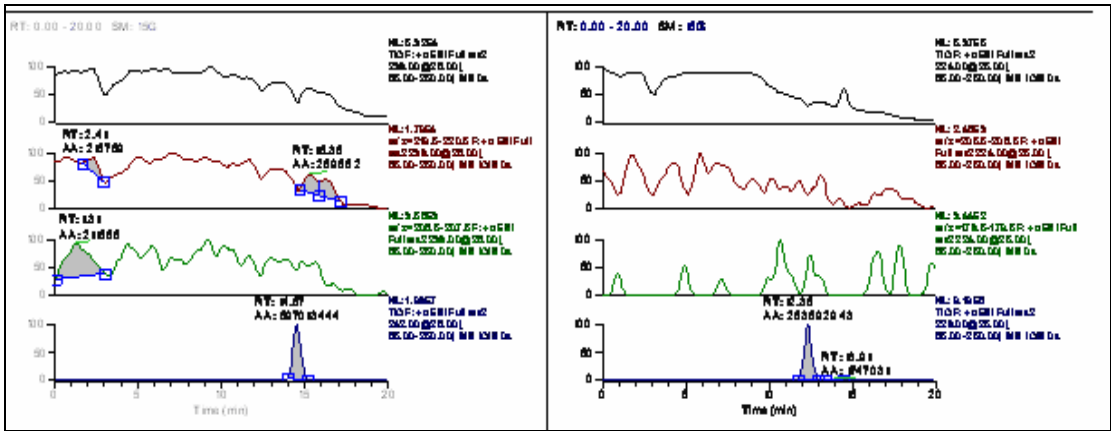
Ketamine is weakly basic (pK_a = 7.5) and exists as a cation at physiological pH. As melanin is a polyanionic polymer, melanin and ketamine interact strongly and facilitate ketamine incorporation into hair. Since concentrations of melanin increase in the order of white < brown < black hair, concentrations of ketamine and norketamine also increase accordingly, as found by Xiang et al. [101]. The present study focused on the development and validation of MIP extraction and, due to the limited number of samples, the connection between hair colour and the concentration of ketamine was inconclusive.

As seen in the chromatograms of (a) the blank extract and (c) the pool sample (Figure 8-14) obtained using the MISPE method, the MIPs led to clean extracts and achieved the aim of this study to provide a cleaner extraction with less interference, as shown in Figure 8-14. From the chromatogram of the pool sample, it seems that most of the interferences were removed during the washing step, leaving only ketamine, norketamine and the internal standards, ketamine-d4 and norketamine-d4 bound to the polymer. Furthermore, the blank analysis demonstrated no ketamine template bleeding.

(a) Blank hair sample



(b) Internal standard ketamine-d4 (left) and norketamine-d4(right)



(C) pooled positive hair samples

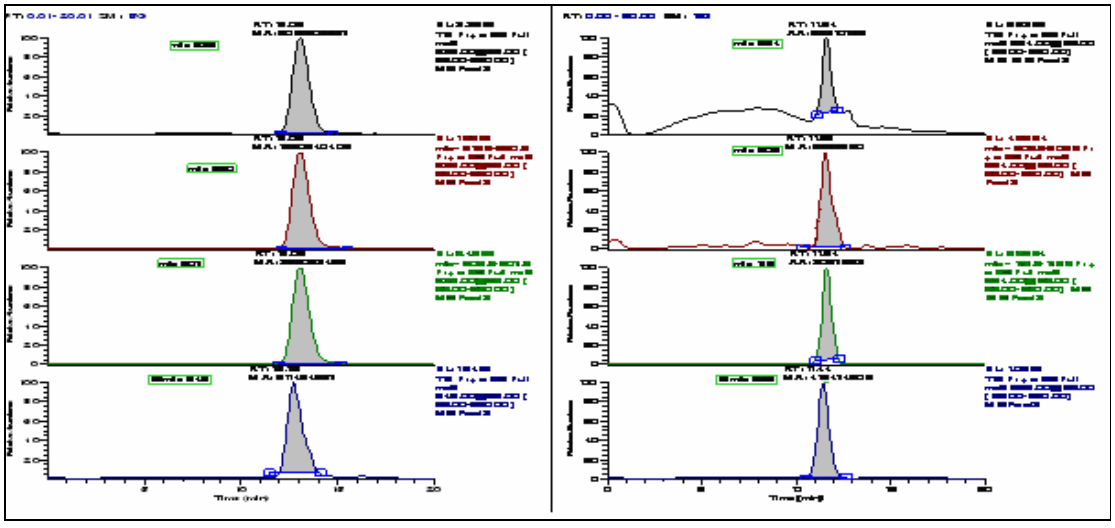


Figure 8-14 LC-MS-MS chromatograms for (a) blank, (b) internal standards and (c) pooled positive hair samples.

The MIP in this study was prepared on a small scale, (1 gram of template) and produced less than thirty columns. Therefore, the MIP columns were reused throughout the study. This demonstrated that the MIP columns were robust and could withstand extreme conditions of different solvents, temperature and pH and may be helpful in decreasing the cost of sample analysis.

8.7 Conclusion

In this study, an MIP extraction method for ketamine in hair samples has been developed and validated. The extraction has been found to be selective and sensitive with less matrix effects than a comparable unpublished method using conventional SPE. The method can also be applied to norketamine, the principal metabolite of ketamine, due to the group-selective binding nature of the MIP, but not to structurally dissimilar analytes such as PCP and tiletamine. The concentrations of ketamine detected in hair samples from chonic ketamine abusers ranged from 0.2 to 5.7 ng/mg and norketamine from 0.6 to 1.2 ng/mg and two samples contained trace amounts of norketamine (below the limit of quantitation).

The MIP columns were found to be reusable, robust and able to withstand various treatments with different pH and solvents as used in this study. The combination of MISPE and LC-MS-MS method detected ketamine and norketamine in hair samples of chronic users. This method may be extended to the detection of single ketamine use such as in drug facilitated rape cases and so may be very useful for the investigation of drug-facilitated crime cases in forensic toxicology.

9 COMPARISON OF MISPE WITH CONVENTIONAL SPE FOR THE DETECTION OF KETAMINE IN HAIR SAMPLES

9.1 Introduction and Aims

SPE methods have been used and accepted for the extraction of ketamine from both urine [218] and plasma [123] samples prior to LC-MS/MS analysis, but have not been well established for the analysis of ketamine in hair using LC-MS/MS. As previously indicated, most work published on the analysis of ketamine and its metabolites were done by GC-MS. In the present study, an SPE procedure was evaluated and validated for comparison with anti-ketamine MIP extraction (MISPE) as described in Chapter 8. MISPE extraction coupled with LC-MS/MS showed good selectivity towards the template, ketamine, and the metabolite, norketamine.

The aim of this study was to compare the ketamine hair results of four hair case samples from Malaysia obtained using a conventional SPE system [217] and the MISPE system developed previously and described in Chapter 8. The study was intended to determine if MISPE showed any advantages or disadvantages over conventional SPE in terms of improved sensitivity, specificity and its potential in reducing matrix effects.

9.2 Experimental

9.2.1 Instrumentation

LC-MS-MS analysis of ketamine was carried out using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose CA, USA) equipped with a Surveyor autosampler and MS pump system. Chromatographic separation was performed using Synergi Hydro RP (150 mm x 2.0 mm ID, 4 µm particle size) from Phenomenex and a 4.0 mm x 2.0 mm, 5 µm guard column (Phenomenex, Torrance CA, USA) with the same packing material as the column. Ionization of analytes was performed using electrospray ionisation (ESI) in the positive ion mode and data were collected in the Selective Reaction Monitoring (SRM) mode and processed using Xcalibur Software version 1.3 also from Thermo Finnigan.

9.2.2 Chemicals and Materials

Methanol, acetonitrile, chloroform, ammonium hydroxide, phosphoric acid, formic acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and ammonium formate were of analytical grade and purchased from BDH (Poole, UK). Ammonium acetate was purchased from Sigma-Aldrich (Dorset, UK).

Ketamine, ketamine-d4, norketamine and norketamine-d4 standards as solutions in methanol were obtained from LGC Promochem (Teddington, UK). World Wide Monitoring Clean Screen® columns (ZSDAU 020) were purchased from United Chemical Technologies, Inc. (Pennsylvania, USA).

9.2.3 Preparation of Solutions

9.2.3.1 Mobile Phase

A mobile phase consisting of 3 mM ammonium formate and 0.001% formic acid in water was prepared by adding 0.189 g ammonium formate and 10 µl of concentrated formic acid to a 1L volumetric flask and making up to 1L with deionised water.

9.2.3.2 Preparation of pH 5.0 Phosphate Buffer

1.70 g of sodium hydrogen phosphate and 12.14 g of sodium dihydrogen phosphate were weighed into a 1 L volumetric flask and made up to volume with deionised water. The pH was adjusted to pH 5.0 using phosphoric acid.

9.2.4 Hair Samples

The four hair samples tested were from chronic users and were obtained with consent from a drug prevention centre in Malaysia. The case samples were screened positive using ketamine Neogen ELISA. Negative hair samples were from volunteers in the Glasgow University laboratory.

9.2.5 Preparation of Hair Samples

Blank hair samples were collected from volunteers in the laboratory. Hair samples were washed with 0.1% aqueous sodium dodecyl sulfate (SDS) and sonicated for 10 minutes. The hair samples were then rinsed and sonicated for 10 minutes, twice with deionised water and twice with dichloromethane, then left to dry in air at room temperature overnight. Each sample was then split

between two separate vials for extraction by SPE and MISPE procedures. Blank hair was also washed using the same procedure prior to spiking with ketamine and norketamine standards to produce the calibration curves.

Spiked hair samples weighing 10 ± 0.1 mg were prepared by adding 1.5 mL 0.1 M phosphate buffer pH 5.0. Standards and internal standards ketamine-d4 and norketamine–d4 were added in the vials prior to the incubation. The vials were left to incubate for 18 hours at 45 ºC. The hair extracts were cooled and transferred into clean vials.

9.2.5.1 Conventional SPE Method

The conventional SPE method selected for this study used a mixed-mode cationic exchange mechanism (Clean Screen® columns, ZSDAU 020). The sorbent is composed of silica substituted with C_8 chains and benzenesulfonic acid residues. Ketamine and norketamine are retained on the column *via* both hydrophobic and ionic interactions.

Clean Screen® (ZSDAU020) extraction cartridges were conditioned sequentially with 3 mL methanol, 3 mL deionised water and 1 mL phosphate buffer (0.1M, pH 5.0). The vortexed samples were loaded in 2 mL phosphate buffer (0.1 M, pH 5.0) and allowed to drip through without application of vacuum. The columns were then washed with 3 mL phosphate buffer (0.1 M, pH 5.0), 1 mL acetic acid (1.0 M) and dried for 5 minutes under full vacuum. The drugs were eluted using 2 mL methanol/2 % aqueous ammonium hydroxide. The eluant was dried under nitrogen at 40^{o}C and the residue was reconstituted in 100 µL of initial composition mobile phase. A 20 µL aliquot was used for LC-MS-MS analysis.

9.2.5.2 Ketamine MIP Extraction Method

MISPE was carried out using the in-house prepared anti-ketamine MIP columns with the optimised procedure described in Chapter 8. Each cartridge was preconditioned with 3.0 mL acetonitrile followed by 0.1 M phosphate buffer pH 5.0. Each sample was passed through a cartridge and washed with 0.5 mL 1% acetic acid in acetonitrile to remove interferences. The analytes retained in the cartridge were eluted with 1.0 mL 30% (v/v) acetic acid in acetonitrile. The eluted samples were blown down to dryness under a stream of nitrogen at 40^{o}C

and reconstituted in 100 µL of the mobile phase in its initial composition. 20 µL was injected for analysis.

9.2.6 LC-MS/MS Conditions

LC was carried out using a mobile phase containing 3 mM ammonium formate and acetonitrile at a flow rate of 0.3 mL min^{-1}. The elution program consisted of a linear gradient (75-25%) of 3 mM ammonium formate over 6 minutes. The percentage of ammonium formate was then decreased to 20% between 6 to 15 minutes. It was held at 10% between 17.0 to 18.0 minutes before being increased to the initial condition (75%) between 18.5 to 25 minutes. All mass spectral data was acquired in electrospray positive ion mode.

The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimized during tuning for each analyte as in Chapter 8. The probe voltage used was 4.5 kV. Internal standard data was collected in selected ion monitoring (SIM) mode and analytes were identified on the basis of their retention time and full MS-MS spectra. The product ion ratios were monitored to gain further qualitative identification data.

9.2.7 Results and Discussion

Hair sample weights, colours and concentrations of ketamine and norketamine detected using the MISPE and SPE procedures are summarised in Table 9-1. The MISPE procedure successfully detected ketamine and norketamine in all four samples Q, R, S and V at concentrations ranging from 0.2-5.7 ng/mg and 0.1 ng/mg to 1.2 ng/mg respectively. The SPE procedure detected ketamine in all four samples at concentrations ranging from 0.5–6.7 ng/mg but only detected norketamine at very low concentrations in samples Q and R and not at all in samples S and V.

Table 9-1 Results of analysis of hair for ketamine and norketamine using MISPE- and SPE-based procedures.

MISPE				
Sample Number	Weight of hair sample (mg)	Hair Colour	Ketamine concentration ng/mg	Norketamine concentration ng/mg
Q	19.96	Dark brown	4.4	present*
R	19.85	Black	5.7	1.2
S	20.02	Black	2.2	0.6
V	10.01	Light brown	0.2	Present*
SPE				
Sample Number	Weight of hair sample (mg)	Hair Colour	Ketamine concentration ng/mg	Norketamine concentration ng/mg
Q	19.92	Dark brown	4.2	present*
R	20.04	Black	6.7	Present*
S	19.82	Black	1.6	Negative
V	10.02	Light brown	0.5	Negative

*** Equal or higher than LOD but below the LLOQ**

When compared to the concentrations of ketamine and norketamine in the pooled samples (a mixed sample of Q, R, S and V) analysed previously (Chapter 8, Table 8-2), the results obtained in this study using MISPE for ketamine and norketamine gave the closest match to those concentrations.

Table 9-2 Mean concentrations of ketamine and norketamine in hair case samples measured individually by MISPE- and SPE-based procedures and in pooled hair case samples.

Mean concentrations in 4 hair samples using the MISPE method (ng/mg hair)		Mean concentrations in 4 hair samples using the SPE method (ng/mg hair)		Mean concentrations in a mixed sample of Q, R, Sand V hair samples (from inter-day precision measurements reported in Chapter 8) (ng/mg hair)	
ketamine	norketamine	ketamine	norketamine	ketamine	norketamine
3.1	0.5	3.3	0.03	2.6	0.8

The results demonstrated the the method using conventional SPE was acceptable for detection of ketamine but was not sensitive enough for detection of

norketamine. The summary of method validation results of the SPE method are shown in Table 9-3 [219].

Table 9-3 Summary of method validation results of the SPE method

Parameters	Ketamine	Norketamine
Linearity	0-10 ng/mg	0-10 ng/mg
LOD	0.2 ng/mg	0.5 ng/mg
LLOQ	0.9 ng/mg	1.8 ng/mg
Recovery	92.3% (%RSD=5.7)	114.2% (%RSD=6.5)
Precision	2.59 % (%RSD= 15.9)	0.74% (%RSD= 22.4)
Matrix Effects	Ion m/z 220 suppressed by 47.3%	Ion m/z 207 enhanced by 62.2%
Hair Samples (ng/mg)		
Q	4.2	< LOD
R	6.7	< LOD
S	1.6	Negative
V	0.5	Negative

Even though higher recoveries were obtained in the SPE method compared to the MISPE method using spiked standards during method validation, the results for real hair samples (Table 9.1) show that extraction steps using MISPE successfully reduced (cleaned up) the matrix effects or magnified (pre-concentrated) the sample compared to SPE and resulted in better detection of norketamine in real hair samples. Better selectivity to ketamine and norketamine using the MIP extraction, as noted before, might be due to the excellent molecular recognition of the template molecule and the lower LODs and LLOQs. The matrix effects in both extraction methods are shown in Table 9-4.

Table 9-4 MISPE versus SPE matrix effects

Analyte	Spiked concentration (ng mL^{-1})	MISPE Matrix Effect (%) [RSD]	SPE Matrix Effect (%) [RSD]
Ketamine	50	- 6.8 [12.3]	-47.3 [9.4]
Norketamine	50	+ 0.2 [13.2]	+ 62.2[16.4]

One more possible reason for lack of selectivity to norketamine by SPE was because two of the hair samples (S and V) were dyed. An analysis using scanning electron microscopy by Guthrie et. al [220] showed some deposition of dye and bleach in hair and these may have caused some interference with the detection of norketamine in the conventional SPE method. Cosmetic treatments such as dyeing reduce the binding of drugs in hair as these processes damage the hair structure, especially cuticle, which protects the inner part of the hair. Ketamine, the parent drug, is more lipophilic and less polar and is more strongly bound in hair than the metabolite, norketamine, which is more hydrophilic and more polar. Norketamine, having less affinity to the hair matrix than ketamine, would be more likely to be washed out of damaged hair than ketamine and this could result in very low concentrations of norketamine in dyed hair.

Hair washings were not analysed in this study, so no conclusions can be drawn concerning possible interferences from the dyed hair samples. The results also demonstrated that MISPE was not affected by interferences from dyed hair samples, due to high selectivity of the imprinted material to ketamine and norketamine and also showed interferences were washed out during the extraction protocol.

The MISPE method had lower LODs (0.1 ng/mg hair) for ketamine and norketamine compared to 0.2 ng/mg and 0.5 ng/mg hair for the SPE method which would have affected the detection of trace levels of norketamine using the SPE method and resulted in negative results for norketamine in samples S and V.

The recoveries of ketamine and norketamine were better using the SPE method, as shown in Table 9-5. However, the MISPE method detected norketamine in all four hair samples compared to the SPE method. This was due to lack of interference and better selectivity based on the excellent recognition of the template molecule of MIP material.

This study was in agreement to an earlier study by Ariffin et al., on the comparison of benzodiazepine analysis in post mortem hair samples using MISPE and SPE, which also detected a higher number of diazepam positive case samples compared to the SPE procedure [132, 221] .

Table 9-5 Extraction recoveries for ketamine and norketamine

Analyte	Spiked concentration (ng 10 mg^{-1})	MISPE Recovery (%) (n=5)	RSD	SPE Recovery (%) (n=5)	RSD
Ketamine	50.00	86.1	9.34	92.3	5.7
Norketamine	50.00	88.4	5.04	114.2	6.5

The average ketamine and norketamine concentrations detected in the pooled positive hair sample by both methods are shown in Table 9-6. The results obtained following MISPE demonstrated higher precision for both ketamine and norketamine than those obtained using SPE, which is also attributed to the low matrix effects obtained using MISPE.

Table 9-6 Inter-batch-precision for MISPE and SPE

Analyte	MISPE (n=3) x 3 different days Pooled samples		SPE (n=3) x 3 different days Pooled samples	
	Mean	R.S.D. (%)	Mean	R.S.D. (%)
Ketamine	2.63	7.9	2.59	15.9
Norketamine	0.87	0.8	0.74	22.4

9.3 Conclusion

MISPE coupled with LC-MS/MS demonstrated good selectivity and sensitivity for ketamine and norketamine in hair. SPE coupled with LC-MS/MS showed higher selectivity to ketamine and norketamine than MISPE in spiked samples but application to ketamine user samples indicated that the MISPE procedure detected more norketamine in all the four samples. High matrix effects in the SPE method might have interfered with the LC-MS/MS analysis and may have interfered with norketamine detection in hair. Both methods detected ketamine at a relatively high concentration in all four hair samples.

The results of this preliminary study strongly suggested that the ketamine MIP can be successfully applied for the detection of ketamine in chronic users. This successful application of MISPE to the detection and quantification ketamine and norketamine in hair from chronic users suggests that the method should be evaluated for its ability to detect ketamine and norketamine in hair after administration of a single dose of ketamine, such as in date-rape cases.

10 AMPHETAMINES

10.1 Introduction

Amphetamine, dextro-amphetamine and methamphetamine are collectively referred to as amphetamines, often referred to as amphetamine type stimulants (ATS). They are members of a large group of synthetic compounds which are similar to the natural substance phenylethylamine, in food such as cheeses and wines, and cathinone, an active ingredient in khat leaves that are chewed in East Africa and the Arab Peninsula for their psychostimulant properties. Naturally-occurring ATS are quickly degraded in the liver by monoamine oxidase, and are not considered to be drugs that are dangerous to health except cathinone which closely resembles the amphetamines and shares the same pharmacology [222].

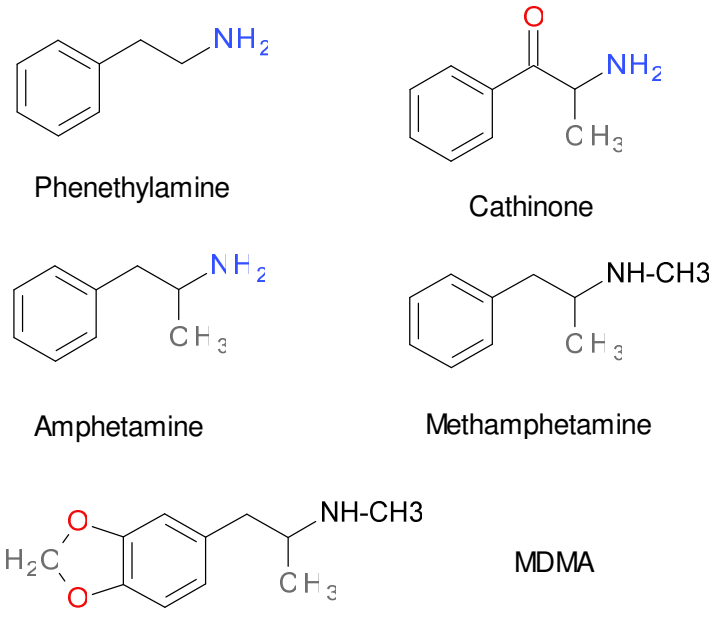


Figure 10-1 Natural products and synthetic amphetamines

Conversely, the synthetic amphetamines such as amphetamine, methamphetamine and 3,4-methylenedioxy-*N*-methylamphetamine (MDMA), which differ from phenylethylamine because of the presence of a methyl group

in the side chain, cannot be degraded by monoamine oxidase and can enter the bloodstream and exert toxic side effects in the body.

Amphetamines have some medicinal uses and the drugs have also been misused around the world throughout most of the 20th Century. Amphetamines are known as psychologically addictive drugs and users often become dependent on them. This is caused by the depression experienced when the drug effects wear off which leads a user taking larger doses frequently to maintain the stimulant "high" effects.

In the United Kingdom, amphetamines are controlled under the Misuse of Drugs Act 1971 as Class B drugs for non-injectable forms and Class A drugs if injectable. They are included in the Regulations to the Act under Schedule 2, by which it is illegal to produce, supply or possess these drugs without a Home Office licence. Unlike Schedule 1 drugs such as cannabis, Schedule 2 drugs have recognised medical uses and can be prescribed by a medical practitioner in the United Kingdom. Also, amphetamines are prescription only drugs under the Medicines Act. Doctors can prescribe pharmaceuticals such as Dexamphetamine Sulphate for narcolepsy or attention deficit hyperactive disorder and this is legal if prescribed and used by the person to whom they were prescribed.

10.2 Chemical and Physical Properties of Amphetamines

Amphetamines occur as stereoisomers as the C2 carbon atom is chiral. The right-handed/dexro-isomer/R(+)-amphetamine is biologically more active than the left-handed/ levo-isomer/S(-)-amphetamine. Chemical modification of the basic amphetamine structure leads to other compounds such as methamphetamine, phentermine, MDMA and hundreds of others which individually possess different pharmacological actions. Many of these chemical derivatives are based on the introduction of substituents on the benzene ring and are known as ring-substituted amphetamines.

10.3 Pharmacokinetics

10.3.1 Routes of Administration

Amphetamines can be administered by a variety of routes and the rates of absorption are different between each route. Oral administrations, including prescription tablets, dissolve in the stomach and are mostly absorbed during passage through the small intestine. This results in gradual absorption and prolonged duration of action as most pharmacy formulations are designed as short-acting and some as time-release medicines. Injection of amphetamines into a vein gives rapid entry into the blood circulation, avoiding an absorption phase, with consequent rapid peak level in the blood and delivery to the brain. Intravenous administration can produce the intense psycho-stimulant effects wanted by the user but have well-known associated risks of infectious disease including HIV and hepatitis. The inhalation route of administration, such as smoking the free base form, can deliver the drug to the enormous surface area of the lungs and ensures rapid absorption and action. Administration by nasal insufflation (snorting) of the salt form also results in rapid absorption through the nasal mucosa.

As mentioned above, the route of administration also affects the legal classification in the United Kingdom. Amphetamines formulated as tablets are Class B under the Misuse of Drugs Act but if prepared for intravenous use they are Class A drugs. Perhaps the most commonly injected drug in the group is methamphetamine, which is the most significant abused drug in the Far East, for example in Japan, where it is traditionally prepared for intravenous administration [227].

10.3.2 Distribution

All amphetamines are highly lipid soluble in their un-ionised state and readily cross the membrane barriers to enter the bloodstream. The plasma half-lives of all amphetamines are in the range 6-12 hours with the exception of methamphetamine, which is 12-24 hours. Amphetamines are concentrated in the kidney, lungs, cerebrospinal fluid and brain. The degree of protein binding and volume of distribution vary widely, but the average volume of distribution is 5

L/kg body weight, which indicates potential problems with respect to post mortem redistribution.

10.3.3 Elimination

Under normal conditions, about 30 % of amphetamine is excreted unchanged in the urine but this excretion is highly variable and is dependent on urinary pH. When the urinary pH is acidic (pH 5.5 to 6.0), elimination is predominantly by urinary excretion with approximately 60% of a dose of amphetamine being excreted unchanged by the kidney within 48 hours. In the situation when the urinary pH is alkaline (pH 7.5 to 8.0), elimination is predominantly by metabolism (deamination) and < 7 % is excreted unchanged in the urine, the half-life ranging from 16-31 hours. For chronic users, amphetamine concentrations in urine range from 1-90 mg/L and methamphetamine from 25-30 mg/L.

10.3.4 Metabolic Pathways of Amphetamines

The amphetamines are metabolised primarily in the liver by three oxidation pathways involving cytochrome P_{450} including N-dealkylation, side-chain hydroxylation and aromatic ring hydroxylation, which are illustrated in Figure 10-2[223]. Additional transformations occur for ring-substituted amphetamines which are described below. Methamphetamine undergoes N-demethylation to give amphetamine or aromatic hydroxylation to form p-hydroxymethamphetamine (pOHMAMP, pholedrine) and is subsequently oxidised to benzoic acid and excreted as glucuronide or glycine (hippuric acid) conjugate. Minor metabolites include p-hydroxyamphetamine (pOHAMP), norephedrine (NOREPH) and p-hydroxynorephedrine (pOHNOREPH) which are pharmacologically active and may contribute to the pharmacological effects in chronic use.

The most commonly-used ring substituted amphetamine, 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) is metabolised by N-dealkylation to 3,4-methylenedioxyamphetamine (MDA), and by O-demethylation to form the intermediates 3,4-dihydroxymethamphetamine (HHMA) and 3,4-dihydroxyamphetamine (HHA). HHMA and HHA undergo O-methylation, forming

4-hydroxy-3-methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA). 3,4-Methylenedioxyethylamphetamine (MDEA) undergoes N-deethylation to form MDA and, following cleavage of the methylenedioxy group, 3,4-dihydroxyethylamphetamine (HHEA) and 4-hydroxy-3-methoxyethylamphetamine (HMEA). The hydroxylated metabolites may also undergo conjugation to form glucuronide and/or sulfate metabolites.

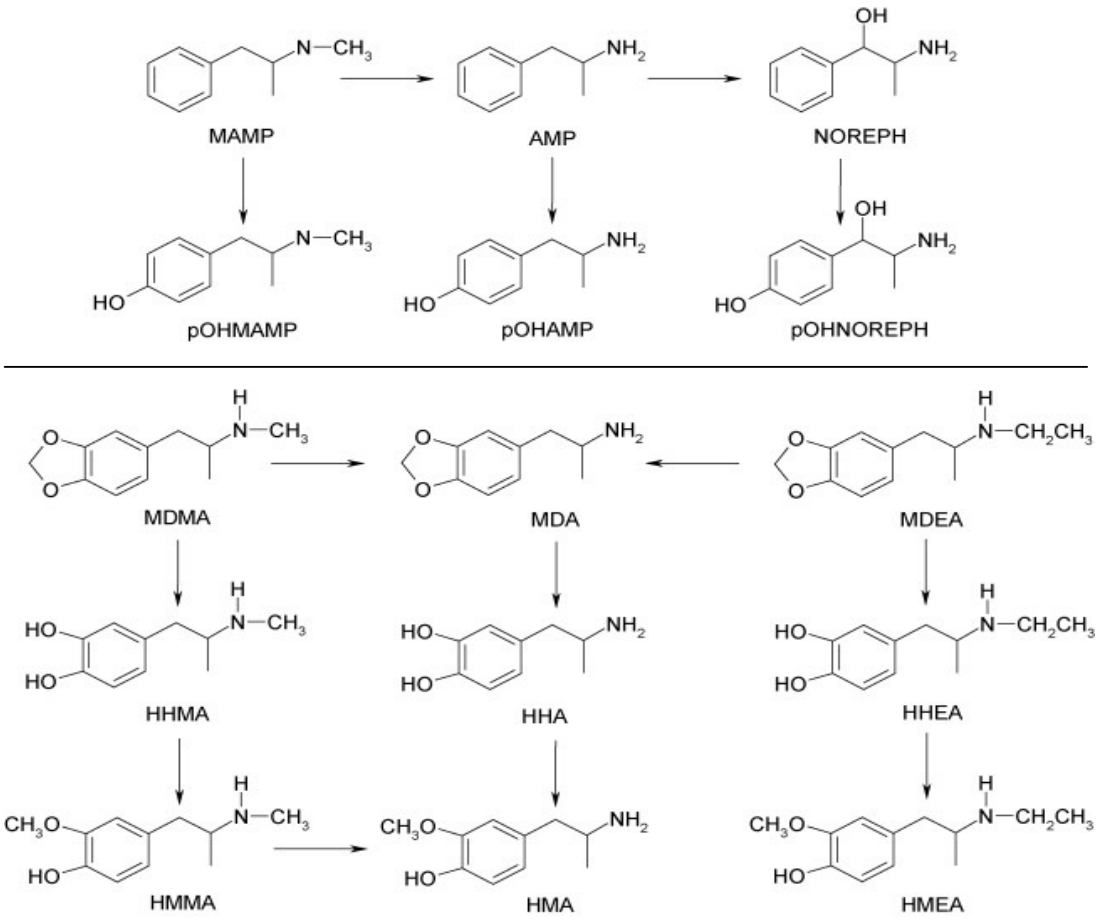


Figure 10-2 Metabolic pathways of methamphetamine (MAMP), amphetamine (AMP), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA). *Abbreviations*: AMP, Amphetamine; HHA, 3,4-dihydroxyamphetamine; HHEA, 3,4-dihydroxyethylamphetamine; HHMA, 3,4-dihydroxymethamphetamine; HMA, 4-hydroxy-3-methoxyamphetamine; HMEA, 4-hydroxy-3-methoxyethylamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MAMP, N-methylamphetamine; MDA, 3,4-methylenedioxyamphetamine; NOREPH, norephedrine; pOHAMP, *p*-hydroxyamphetamine; pOHNOREPH, *p*-hydroxynorephedrine; pOHMAMP, *p*-hydroxymethamphetamine.

10.4 Pharmacodynamics

10.4.1 Mode of Action

Amphetamines are sympathomimetic drugs which stimulate both the central and peripheral nervous system. They may be direct agonists at norepinephrine (noradrenaline) receptors but may also increase the rate of release of norepinephrine, the neurotransmitter of postganglionic sympathetic nerve fibres. Increased central activity in the brain acts on the hypothalamus-pituitary-adrenal axis which causes epinephrine to be released from the adrenal medulla into the circulation. Epinephrine stimulates the sympathetic part of the autonomic nervous system, which increases heart and respiration rates and alters the diameter of blood vessels so that oxygenated blood is preferentially supplied to the muscles and brain, such that mental alertness is increased. The digestive system is inhibited to leave more blood for muscle use and this decreases appetite.

10.4.2 Effects of Amphetamines

An amphetamine user may experience a temporary boost in self-confidence and feel far more energetic than usual, talkative and can perform increased physical activity, but may find it difficult to rest and sleep and will have little or no appetite.

10.4.3 Side effects

After a period of chronic use, an amphetamine user becomes exhausted physically and mentally due to extensive physical activities, loss of water and inability to sleep. As a result, users often feel fatigued and depressed for a few days after using amphetamines and may also feel irritable, anxious and restless. Other side effects include blurred vision and dizziness. Many women who use amphetamines find that their periods become irregular or even stop. High doses, especially if frequently repeated, can produce delirium, panic attacks, hallucinations and feelings of paranoia. Acute overdose of amphetamines produces seizures, hypertension, tachycardia, hyperthermia, psychosis, hallucinosis, stroke and may result in a fatality.

10.5 Medicinal Use of Amphetamines

Amphetamine was first marketed in the 1930s under the trade name Benzedrine, an over-the-counter inhaler used to treat nasal congestion. Later, in the 1930s and 1940s, both dextroamphetamine (Dexedrine) and methamphetamine (Methedrine) were used by the armed forces as performance enhancers in World War II. Amphetamines were common in the 1950's and 1960's for treatment of mild depression and as anorexics for body slimming. Their current medical use is limited, with only Dexedrine (dexamphetamine sulphate) currently available for use in the treatment of narcolepsy - a rare disorder marked by episodes of uncontrollable sleep and methylphenidate (Ritalin) for the treatment of attention deficit hyperactivity disorder (ADHD) in children.

10.6 Misuse of Amphetamines

According to the World Drug Report 2006 published by UNODC [224], 25 million people used ATS in 2004, of which some 10 million people used ecstasy either recreational or regularly. More than 60 per cent of the world's ATS users live in Asia while more than 50 per cent of the world's ecstasy users live in Western Europe and North America. Annual prevalence of amphetamine use is highest in Oceania, followed by North America and East and South-East Asia.

In Europe, which is home to an estimated 2.7 million users of ATS, amphetamine use is far more widespread than the use of methamphetamine which is limited to the Czech Republic and some Baltic States. There was a small fall in the reported use of amphetamine in UK from 1996 to 2000 after years of increases in the early 1990s. This is also reflected in the report by UNDOC which indicates that the markets for ATS seem to be stabilizing, reflecting improved international law enforcement cooperation and improvements in precursor control.

10.7 Toxicological Data

The toxic dose of an amphetamine derivative varies considerably due to individual variations and the development of tolerance. Fatal amphetamine cases have been reported following ingestion of doses as low as 1.3 mg/kg (91

milligrams for a person weighing 70 Kg), although tolerance has been developed to accommodate doses of up to 1,000 mg at a time and up to 5 g in a day, with a blood levels from 2.0 to 3.0 mg/L. The recreational dose of MDMA or MDEA is of the order of 80-120 mg and produces blood levels in the range of 0.1– 0.25 mg/L. However, most cases of serious toxicity or fatality have involved blood levels ranging from 0.5 mg/L to 10 mg/L, up to 40 times higher than the usual recreational range, although some have had levels as low as 0.11– 0.55 mg/L which are in the normal range or a little above it. This is an important point, because it demonstrates the degree to which the seriousness of the effects can be dependent on environmental factors other than the drug concentration [225].

10.8 Previous Work and Aims

There are numerous publications on analytical procedures for the detection and identification of amphetamines in biological fluids including methods to detect amphetamines as a group in a variety of matrices, such as urine[226], plasma [227], brain and other tissues [228,229], sweat [230, 231,232], meconium [223], oral fluid [31,232] and skin [233]. Gas chromatography-mass spectrometry has dominated the methods for determination of amphetamines because of the volatile nature of the analytes, especially following the preparation of chemical derivatives.

A few papers deal with the analysis of amphetamines using liquid chromatography-mass spectrometry [127,234-237]. One paper describes the analysis of amphetamines which were successfully extracted using a commercial molecularly imprinted polymer prior to LC-MS/MS analysis, which was the aim of this study [238] .

11 APPLICATION OF MISPE AND LC-MS/MS FOR THE DETERMINATION OF AMPHETAMINES IN WHOLE BLOOD

11.1 Introduction

The company MIP Technologies has developed proprietary products based on molecularly imprinted polymers to improve the extraction of analytes from complex matrices. The products are based on the selectivity of the MIPs which are capable of extracting a wide range of analytes or analyte classes.

The products are based on selectivity which is introduced during MIP synthesis, in which a template molecule, which is either the target analyte or one designed to mimic the analyte, guides the formation of specific cavities that are sterically and chemically complementary to the target analyte(s), as described in detail in Chapter 6.

Molecularly imprinted polymers have been used successfully to extract several analytes, such as clenbuterol, a drug prescribed as a decongestant and bronchodilator for breathing disorders [239], chloramphenicol, an antibiotic [240] and ß-blockers, a class of drugs used for the management of cardiac arrhythmias, heart failure and hypertension [241]. Recently, MIPs have been used successfully for extracting zidovudine and stavudine, which are used to treat acquired immunodeficiency syndrome (AIDS), from plasma samples [242].

In this study, Amphetamines SupelMIP™, a commercial molecularly imprinted solid-phase extraction product for amphetamine-type compounds was evaluated for the extraction of amphetamines from post-mortem blood. Extraction protocols using Amphetamines SupelMIP™ have previously been used to obtain high recoveries for amphetamines from urine [238].

11.2 Experimental Section

11.2.1 Instrumentation

A Surveyor HPLC system linked to an LCQ™ Deca XP Plus ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was used for analysis of

whole blood samples. The equipment came with a data processing system, Xcalibur 1.3. HPLC was performed on a Synergi Hydro RP column (150 mm x 2.0 mm i.d., 4 µm particle size, Phenomenex, Torrance CA, USA) fitted with a 5 µm guard column with the same packing material as the column. The guard column holder was from Phenomenex, Macclesfield, UK.

11.2.2 Chemicals and Materials

Amphetamine, methamphetamines, MDMA, MDA and MDEA and their deuterated internal standards, amphetamine-d11, methamphetamines-d14, MDMA-d6, MDA-d5 and MDEA-d6 were obtained from LGC Promochem (Teddington, UK). Ammonium acetate and ammonium formate were purchased from Fluka (Buchs, Switzerland). Formic acid, methanol and acetonitrile were of HPLC grade and were from BDH (Poole, UK). Amphetamines SupelMIP^{TM} columns (25 mg/3 mL, lot number SPMG060961) were purchased from Supelco (Bellafonte, PA). Vials and caps for sample injection using an autosampler were obtained from Kinesis Ltd, (Greensbury Farm Bolnhurst, UK).

11.2.3 Standard Solutions

Stock solutions of all standards, at a concentration of 1.0 mg/mL, were diluted to obtain working solutions of 10 ng/µL, 1.0 ng/µL and 0.1 ng/µL. Stock solutions of internal standards, 100 ug/mL were also diluted to obtain working standards at a concentration of 1.0 ng/µL. These working solutions were used to construct calibration curves with six points at concentrations of 0, 25, 100, 250, 500 and 1000 ng/mL for the 5 types of amphetamines. Stock and working solutions were kept refrigerated at 4 °C when not in use, with a nominal shelf life of 6 months.

11.2.4 Blank Blood

Time-expired packed red blood cells were obtained from the blood bank (Scottish Blood Transfusion Service, Gartnavel Hospital). These red blood cells were suspended in an equal volume of isotonic saline (0.1 M sodium chloride solution) before use. Ethical approval for use of the blood cells was obtained from the Scottish Blood Transfusion Service, Edinburgh.

11.2.5 Solutions

11.2.5.1 Preparation of Mobile Phase

An acidic mobile phase consisting of 3 mM ammonium formate and 0.001% formic acid in water was prepared by adding 0.189 g ammonium formate and 10 µl of concentrated formic acid to a 1L volumetric flask and making up to 1L with deionised water.

11.2.5.2 Preparation of Ammonium Acetate Buffer (10 mM, pH 8.0)

In a fume hood, 0.575 mL glacial acetic acid was measured into a 1000 mL volumetric flask containing 800 mL deionised water. Then 1.30 mL concentrated ammonium hydroxide was added. The pH was adjusted to 8.0±0.1 with ammonium hydroxide if the pH was less than 8.0 or acetic acid if the pH was greater than 8.0. The solution was then made up to 1.0 L with deionised water.

11.2.5.3 Preparation of Other Solvents

Aqueous acetonitrile (60:40 v/v) was prepared by adding 60.0 mL acetonitrile to 40 mL deionised water. 1 % HAc (v/v) was prepared by adding 1.0 mL glacial acetic acid to 99 mL acetonitrile. 1 % formic acid in acetonitrile (v/v) was prepared by adding 1.0 mL concentrated formic acid to 99.0 mL MeCN.

11.2.6 LC-MS-MS Conditions

LC analysis was carried out using a mobile phase gradient programme combining 3 mM ammonium formate buffer + 0.001% formic acid (pH ~3) and acetonitrile at a flow rate of 0.3 mL/min as shown in Table 11-1. A 20 µl aliquot of sample was injected onto the Synergi Hydro RP LC column using partial loop mode.

Table 11-1 Gradient conditions

Time (min)	3 mM ammonium formate + 0.001% formic acid (A %)	Acetonitrile (B %)	Flow rate ($\mu L.min^{-1}$)
0	75	25	300
6	75	25	300
15	20	80	300
17	10	90	300
18	10	90	300
18.5	75	25	300
25	75	25	300

Mass spectral data were acquired for both analytes and their deuterated internal standards in the electrospray (ESI) positive ion mode which produced protonated molecular ions, $[M + H]^{+\cdot}$ The electrospray probe voltage used was 4.5 kV. The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimized during tuning for each analyte (Table 11-2).

Internal standard data was collected by Selected Ion Monitoring (SIM) for identification of the parent ions and analyte data was collected in the mode over the mass range m/z 60-250. Selected reaction monitoring (SRM) was used where one parent ion and one product ion were identified because of the low molecular weight of these compounds [243]. The quantitation ion was the major product ion produced on precursor fragmentation. The ratios of quantitation ion to internal standard were calculated. Therefore in this study for positive sample identification the ratio of quantitation ion to internal standard was used, the value either greater than or within ± 20 % of the ratio for the lowest calibration standard.

The optimum tuning LC parameters, the precursor and the product ions for amphetamines was shown in Table 11-2.

Table 11-2 The optimum tuning LC parameters, the precursor and the product ions for amphetamines

Compound	Sheath Gas (AU)	Auxiliary Gas(AU)	Capillary temperature (°C)	Collision Energy (%)	Precursor Ion	Product Ion
Amphetamine	15	5	210 *	23	136	119
Methamphetamine	15	5	210	28	150	119
MDA	15	15	220	21	180	163
MDMA	15	15	280	25	194	163
MDEA	15	15	220	20	208	163

*** 210 °C was used in the LC-MS method**

11.2.7 Extraction method

11.2.7.1 Sample Pre-treatment

Whole blood is a complex matrix containing many proteins which can subsequently cause matrix effects during the MIP extraction process. For this reason, a pre-treatment step was applied. Standards and internal standard were spiked into 1.0 mL aliquots of blood which was then diluted with 1.0 mL ammonium acetate buffer, pH 8.0, vortexed mixed and centrifuged at 3000 rpm for 10 minutes. The supernatant was transferred to a clean tube for the loading step in the extraction process.

11.2.7.2 MISPE Protocol

Amphetamines SupelMIP^{TM} SPE cartridges were used to extract the amphetamines from blood. The SPE protocol was based on the manufacturer's suggestion. It was assumed that the molecularly imprinted polymer material in the columns was made by monolith polymerisation as the particles varied in size, with an average particle size of 58.6 µm. The column was first conditioned with 1.0 mL methanol, followed by 1.0 mL 10 mM ammonium acetate buffer, pH 8.0

without the application of a vacuum. Then the pre-treated blood samples were loaded on the SPE cartridges. The cartridges were then washed sequentially with 2 x 1.0 ml DI water, taking care not to let the column dry out, 1.0 mL 60/40 v:v MeCN/DI water followed by a drying step of 5-10 minutes with full vacuum, and finally with 1.0 mL of 1 % HAc in MeCN. 2 x 1.0 mL 1 % formic acid in MeCN was used to elute the amphetamines with mild application of vacuum between each elution. The combined SPE eluant was evaporated to dryness under a stream of nitrogen gas without heating. Finally, the residues were reconstituted with 100 µL initial mobile phase and vortex mixed.

11.2.8 Method Validation

11.2.8.1 Linearity, LOD and LLOQ

Linearity was established for 5 amphetamines over the range 0-1000 ng/mL. Amphetamines were spiked into human whole blood to achieve concentrations of 0, 25, 100, 250, 500, and 1000 ng/mL along with internal standards at 50 ng/mL (n=2) and these were then extracted by MISPE and analysed by LC-MS/MS. Calibration curves were prepared by plotting peak area ratios of standards/internal standard against the spiked analyte concentrations. These were subjected to linear regression analysis. Limit of Detection (LOD) values were calculated statistically using three times the standard error of the regression line and Lower Limit of Quantification (LLOQ) values were calculated statistically using ten times the standard error of the regression line [29, 244, 245].

11.2.8.2 Matrix Effect Assessment

This study was conducted to assess the effects on the LC-MS analysis of the extracts of interferences which are co-extracted along with the analytes from the blood matrix. Three replicates of 1 mL blank blood were spiked at three levels (50, 450 and 900 ng/mL) with amphetamine, methamphetamine, MDMA, MDA and MDEA. Another three replicates were prepared by spiking the same amounts of amphetamines in 2 mL of loading buffer rather than blood.

All of the samples were vortexed, centrifuged and extracted using the MISPE procedure described earlier. 50 ng of each internal standard was added to each replicate after the extraction. The peak area ratios of the analytes to internal

standards in blood extracts were divided by those obtained from samples prepared using the loading buffer to give the matrix effects as a percentage [33].

11.2.8.3 Recovery studies

Amphetamines were spiked at concentrations of 50, 450 and 900 ng/mL of each compound in 1 mL aliquots of blood (n=6), which were then processed using the MISPE procedure. Two unextracted standards of each concentration levels were also prepared and were kept in the fridge throughout the extraction. 50 ng of deuterated internal standards of each compound were then added after MISPE to each extracted and unextracted standard before blowing down under nitrogen. Percentage recoveries were determined by comparing peak area ratios of the extracted and unextracted standards.

11.2.8.4 Intra- and Inter-day Precision

The intra- and inter-day precisions were determined by analysing human whole blood samples spiked at concentrations of 50, 450 and 900 ng/mL with amphetamine standards six times in the same day and once a day during 3 successive days.

11.2.8.5 Application to Case Blood Samples

Case blood samples were obtained from the Department of Forensic Medicine and Science, University of Glasgow and were analysed using the method protocol described earlier. The results obtained by LC-MS/MS method in this study were compared with those previously obtained during the initial investigation of the cases using the accredited Departmental GC-MS procedure (without reanalysis) .The time period between the samples being analysed for the first time and reanalysed using MISPE extraction and the developed LC-MS/MS method was six months to a year.

11.3 Results and Discussion

11.3.1 Linearity and Determination of the LOD and LOQ

Regression analysis of graphs of peak area ratios of amphetamines to internal standards versus concentration showed good linearity in the range of 0-1000 ng/mL as shown in Figure 11-1.

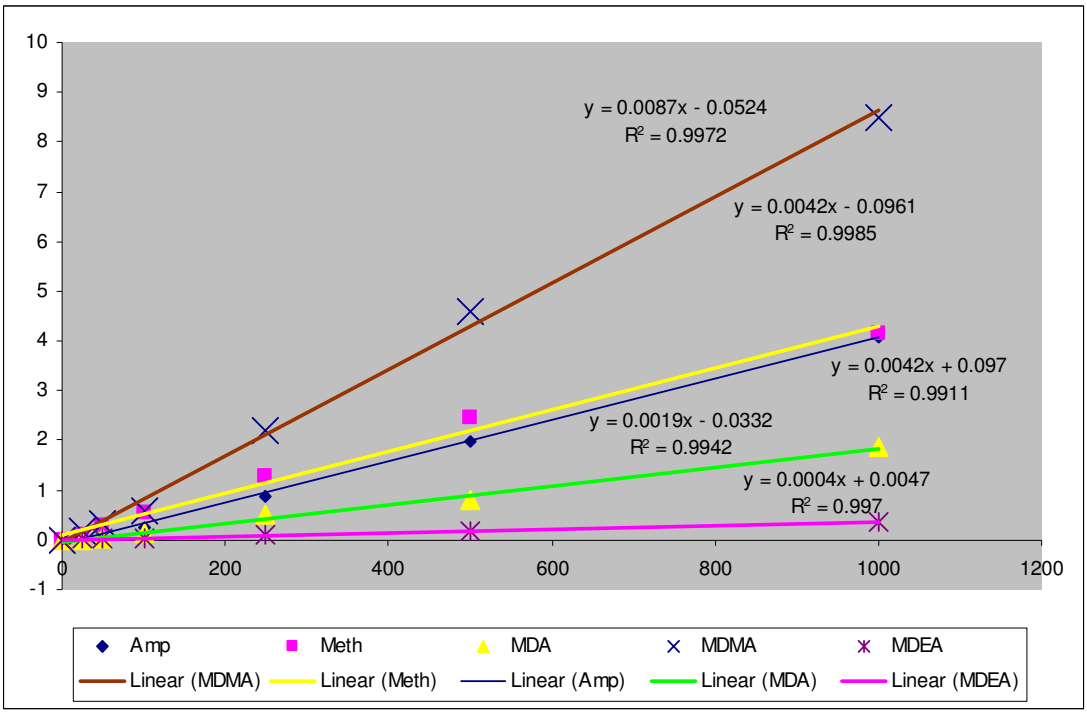


Figure 11-1 Calibration curves for the 5 amphetamines

Correlation coefficients (R^2) for the calibration curves were better than 0.99 in each case. Calculated LODs and LLOQs based on regression lines for the 5 amphetamines are shown in Table 11-3.

Table 11-3 Calibration curve regression coefficient, linear range, LOD and LLOQ for five amphetamines in whole blood and comparison of LOD/LLOQ with a validated GC-MS method

Amphetamine	Coefficients Variations R^2	Linear range ng/mL	LC-MS/MS LOD ng/mL	GC-MS LOD ng/mL	LC-MS/MS LLOQ ng/mL	GC-MS LLOQ ng/mL
Amphetamines	0.9972	0-1000	0.4	10	1.3	25
Methamphetamine	0.9985	0-1000	0.2	10	0.8	25
MDMA	0.9911	0-1000	0.3	1	0.9	5
MDA	0.9942	0-1000	0.4	1	1.4	5
MDEA	0.9970	0-1000	0.6	5	1.9	10

MISPE has been found in previous studies to provide lower detection limits than conventional SPE due to increased selectivity which results in cleaner extracts and in this study the application of the method to the whole blood samples gave low LODs (<1.0 ng/mL) and LLOQs (<2.0 ng/mL) for all five amphetamines investigated. This compares favourably with the routine GC-MS procedure, which has LODs and LOQs in the range 1.0 ng/mL and 25.0 ng/mL respectively.

11.3.2 Matrix Effect Study

The matrix effects observed during the analysis of 5 amphetamines at three concentrations are shown in Table 11-4.

Table 11-4 Matrix effects for 5 amphetamines

Analyte	Spiked concentration (ng mL^{-1})	Matrix Effect (%) [RSD]
Amphetamine	50	- 4.9 [2.4]
	450	+ 4.6 [6.6]
	900	- 4.0 [9.9]
Methamphetamine	50	+10.3 [0.9]
	450	+7.3 [8.4]
	900	+6.3 [11.1]
MDMA	50	+3.9 [6.7]
	450	+0.2 [5.0]
	900	+0.1 [2.6]
MDA	50	+12.3 [0.6]
	450	+5.0 [9.3]
	900	-7.2 [3.3]
MDEA	50	-5.0 [2.1]
	450	-5.9 [5.4]
	900	-5.0 [9.1]

From the data given in Table 11-4, it can be seen that blood matrix components co-extracted by MISPE were found to cause acceptable levels of suppression and enhancement to the MS response during LC-MS analysis of the five amphetamines. Good MS compatibility and minimized matrix effects were obtained due to elimination of interfering substances by the wash steps included in the MISPE protocol. The cartridges were washed sequentially with 2 x 1.0 mL deionised water, 1.0 mL 60/40 v/v MeCN/DI water followed by a drying step of 5-10 minutes with full vacuum, and with 1.0 mL of 1 % HAc in MeCN. The results demonstrated that there were no significant interfering peaks which co-eluted at the retention times of the analytes and internal standards, demonstrating again the improvement in selectivity brought by the MIP that allows the removal of these interferences. Subsequently, all standard solutions were prepared in whole blood to match the matrix of real case samples.

11.3.3 Recovery Study

Recoveries of amphetamines from blood at different concentrations are presented in Table 11-5. Overall the highest recovery was obtained for methamphetamine, followed by MDMA, amphetamine, MDA and MDEA.

Table 11-5 Recoveries of amphetamines from human whole blood

	50 ng/mL		450 ng/mL		900 ng/mL	
QA compound	Mean recovery (%) (n=6)	RSD of recovery (%)	Mean recovery (%) (n=6)	RSD of recovery (%)	Mean recovery (%) (n=6)	RSD of recovery (%)
Amphetamines	40.9	8.1	38.3	7.8	40.2	4.5
Methamphetamines	42.2	7.6	61.4	8.2	50.3	1.2
MDA	38.0	13.4	37.9	9.0	38.5	9.8
MDMA	47.5	8.5	50.3	9.5	50.1	4.8
MDEA	32.4	10.6	33.7	8.2	31.4	9.6

The recoveries were in the range 32.4 % to 61.4 % with RSDs of less than 11 %. These were significantly lower than the recoveries from urine found in a study performed by the manufacturer using the same MISPE columns and extraction protocol but different LC column for separation. The manufacturer's recoveries were between 97 % and 113 % with % RSD of 1.41 to 9.84 for the same group of amphetamines analysed in urine [238]. The comparison GC-MS method did not provide recovery data for the validated method but data for drift assay showed no drift in analytes concentration.

According to Martin et al., in order to get high efficiency results in the analysis of beta-blockers in plasma samples using MIP, it was necessary to perform a protein precipitation step ahead of the MISPE [246]. Therefore unsatisfactory recoveries in this study may be due to the incomplete protein precipitation step ahead of the MISPE protocol used for whole blood in this study. However, the method was suitable for routine application as the LODs and LOQs and precision (Section 11.3.4) were good. The average recoveries from blood were not significantly different for the 5 amphetamines at three levels according t values calculated using Student's t-test (Table 11-6).

Table 11-6 T-test results

Set of samples	Set A	Set B	Set C
Concentration level	**50 ng/mL**	**450 ng/mL**	**900 ng/mL**
Amphetamine	40.9	38.3	40.2
Methamphetamine	42.2	61.4	50.3
MDA	38	37.9	38.5
MDMA	47.5	50.3	50.1
MDEA	32.7	33.7	31.4
T test results	**Set A vs. Set B**	**Set A vs. Set C**	**Set B vs. Set C**
T value	0.74	0.68	0.73
P value	<0.05	<0.05	<0.05

11.3.4 Intra- and Inter-day Precision

The intra- and inter-day precisions were determined by analysing human whole blood samples spiked with amphetamines at concentrations of 50, 450 and 900 ng/mL analytes, ten times in the same day and once a day during three successive days.

Good precisions were obtained using the method described above. Relative standard deviations (RSDs) for intra- and inter-day precision were less than 10 % and are shown in Table 11-7.

Table 11-7 Intra- and inter-day precisions for amphetamines in human blood samples

Amphetamines	Amount added (ng/mL)	Intra-day (n=6) Amount detected (ng/mL)	RSD (%)	Inter-day (n=6 x 3) Amount detected (ng/mL)	RSD (%)
Amphetamine	50	52.9	5.8	52.6	6.2
	450	449.5	1.7	446.5	0.6
	900	896.5	1.1	897.8	0.2
Methamphetamine	50	54.7	2.6	51.3	6.0
	450	452.2	1.9	448.9	0.7
	900	895.6	0.8	900.5	0.6
MDA	50	54.7	0.6	54.5	1.7
	450	446.2	1.7	446.6	1.9
	900	895.4	0.4	897.3	0.5
MDMA	50	52.7	1.5	48.7	7.7
	450	447.4	1.1	446.8	0.9
	900	897.2	0.4	892.6	1.0
MDEA	50	50.4	5.3	50.8	5.1
	450	447.3	7.2	450.8	8.1
	900	903.4	8.5	896.9	1.1

The method showed good reproducibility as obtained in the intra- and inter-day precision results. The precision values for a comparative GC-MS method are tabulated in Table 11.8 for comparison.

Table 11-8 The Intra- and inter-day precisions for amphetamines in human blood samples for GC-MS method

Amphetamines	Amount added (ng/mL)	Intra-day (n=10) Amount detected (ng/mL)	RSD (%)	Inter-day (n=10 x 10 days) Amount detected (ng/mL)	RSD (%)
Amphetamine	125	128.9	1.6	127.9	6.2
Methamphetamine	125	111.8	6.7	121.4	8.1
MDA	125	138.6	2.2	124.8	9.7
MDMA	125	125.5	2.6	116.5	8.2
MDEA	125	132.7	2.3	123.30	7.0

11.3.5 Case Samples

Blood samples were obtained with permission from post mortem cases from Forensic Medicine and Science, University of Glasgow which had previously been analysed and reported and which were due for disposal. Analysis of case materials is permitted as long as the results of analysis are given to the pathologist and Procurator Fiscal to supplement the case investigation.

The samples were selected from positive and negative cases for the 5 amphetamines and the results were compared with the routine GC-MS method. It was not possible to obtain samples which were positive for each of the 5 amphetamines. Most of the samples were positive only for amphetamine and one case was positive only for MDMA. Drug related deaths caused by amphetamines in Scotland are not many, as shown in the statistics, and there were only 13 drug related deaths involving ecstasy in 2006 [218].

The results for real cases are shown in Table 11-9. In this study the application of commercial MISPE successfully detected amphetamines in the positive samples but the concentrations found were lower than those obtained using the accredited routine GC-MS method. This is unlikely to be due to the low recoveries of amphetamines of this method, which were between 30-65 % for the

5 amphetamines at all levels, as this is corrected by the use of internal standards. It is possible that analyte losses occurred during storage between the times of the original analysis and the present study. Even though amphetamines are very stable compounds, studies of the stability of amphetamines have shown that MDA, MDMA and MDEA have some degradation when stored at 4° C for more than 13 weeks especially at low concentrations [21].

On the other hand, the method detected low levels of analytes in the samples such as for MDMA and has proven the method has low LODs and LLOQs for the 5 amphetamines. Of two negative samples from GC-MS one also came out negative for all five analytes in the MIPSE-LC-MS/MS analysis but the other showed the presence of MDMA which was found negative in the original analysis.

Table 11-9 Comparison of GC/MS and LC/MS results

Sample Number	GC-MS		LC-MS/MS	
	Analyte	Concentration (ng/mL)	Analyte	Concentration (ng/mL)
1	Amphetamine	380	Amphetamine; MDMA	116 6.5
2	Negative for all 5 amphetamines		Negative for all 5 amphetamines	
3	Negative for all 5 amphetamines		MDMA	9.9
4	Amphetamine	860	Amphetamine	529
5	Amphetamine	870	Amphetamine	335
6	MDMA	1800	MDMA	1548
7	Amphetamine	890	Amphetamine	636

It was also noted that most of the cases were from poly-drug users and not from users of amphetamines alone (Table 11-10). Apart from amphetamines, the common drugs present in the samples were benzodiazepines, cocaine and metabolites, morphine and metabolites, cannabis and alcohol. Poly-drug use was also common in England as discussed by Williams and Parker in their published paper [247] .

Table 11-10 Other drugs present in the post mortem blood samples

Sample Number	Other drugs detected (mg/L)
1	Diazepam, temazepam, lamotrigine, desmethyldiazepam, oxazepam, benzoylecgonine, alcohol
2	negative for all drugs
3	negative for all drugs
4	Carboxyhaemoglobin (19 %), alcohol
5	Codeine and metabolites, cannabis and metabolites, benzodiazepines
6	Alcohol, lignocaine, diazepam, lorazepam, phenytoin
7	Benzodiazepines, morphine and metabolites, mirtazapine, alcohol

11.4 Conclusion

A commercial cartridge was evaluated for use in a method for the quantitation of five amphetamines in human blood based on MISPE and LC-MS/MS analysis. The method demonstrated good linearity, LOD, LOQ, accuracy and precision and low matrix effects. However the recoveries obtained were lower than expected for the five amphetamines and the method requires further optimisation for routine use in forensic toxicology if lower detection limits of drugs in whole blood are required. The recoveries for the five amphetamines were lower than in the comparison GC-MS method but the LODs and LLOQs were found to be better and suitable for detection of low levels amphetamines in post mortem blood. This may be a result of the incomplete sample pre-treatment ahead of the MISPE protocol used for whole blood in this study, and may be affected by the gap between the original analyses and samples being reanalysed in this study (within six months to a year) during which some degradation of the analytes may have occurred in the whole blood samples.

12 GENERAL CONCLUSIONS AND FURTHER WORK

12.1 General Conclusions

In response to the challenge in the field of forensic toxicology to improve methods of identification and quantification of analytes, particularly in terms of selectivity and sensitivity, the work in this thesis investigated the use of a novel technique in sample preparation, molecularly imprinted solid phase extraction (MISPE). The present work also investigated the use of LC-MS/MS analysis which has recently become available in routine forensic toxicology for the identification and quantification of drugs in biological specimens. The model compound used was ketamine, for which an anti-ketamine MIP was synthesised in-house, and amphetamine, for which a commercial MIP was used. During the period of this research, the majority of published work regarding the analysis of ketamine and amphetamine was still based on GC-MS.

In the present work, LC-MS/MS analysis following ketamine MIP extraction was found to be selective and sensitive and to give clean extracts with fewer matrix effects than a comparable method based on conventional SPE. The anti-ketamine MIP had cross-reacted with norketamine, the main metabolite of ketamine due to the group-selective binding nature of the MIP. The MIP columns were found to be reusable, robust and able to withstand treatment with a range of different pH values and solvents. In addition, preliminary results indicated that chiral selectivity may also be obtained using an anti-S-ketamine MIP.

The combination of MISPE and LC-MS-MS detected ketamine and norketamine in a higher proportion of hair samples of chronic users compared to a conventional SPE method. For this reason, the MISPE method should be extended for more challenging analysis of detecting single ketamine use, such as in drug facilitated rape cases, which would be very useful for the investigation of these cases in forensic toxicology.

The commercially-available product, Amphetamines SupelMIP™, was evaluated for use in MISPE and LC-MS/MS analysis for quantitation of five types of amphetamines in human post mortem blood. The method demonstrated good linearity, LOD, LLOQ, accuracy, precision and matrix effects but the recoveries obtained were lower than expected for the five amphetamines and therefore required further optimisation to trigger the selectivity of the MIP before being recommended for use in forensic toxicology, which requires high recoveries in order to permit the detection of drugs at low concentrations in whole blood.

The main problem arising from the in-house synthesised MIPs was due to bleeding of entrapped template. A vigorous and time-consuming procedure was carried out to extract the template completely from the polymer. In the ketamine MIP, the template strongly interacted with the monomer and was only removed after washing more than 14 times with an appropriate extraction solvent. However, for Amphetamines SupelMIP™, this problem was solved by the careful bleeding test by the manufacturer.

The present work also included the development and validation of an ELISA screening method and an LC-MS/MS confirmation method for the identification and quantitation of ketamine and its major metabolite norketamine in urine samples. The Neogen® ELISA kit was found to be sensitive, specific and precise for ketamine screening at a cut-off concentration of 25 ng/mL coupled with an LC-MS/MS cut-off of 2 ng/mL. The ELISA procedure demonstrated 100 % cross-reactivity to ketamine and minimal cross-reactivity to its main metabolite norketamine. The LC-MS/MS confirmation method demonstrated excellent linearity, LOD, LLOQ, accuracy and precision, with acceptable matrix interference effects. The screening efficiency of ELISA and the LC-MS/MS confirmation method was evaluated with 34 urine specimens from ketamine users collected from persons attending pubs and nightclubs in Malaysia. The combination of tests demonstrated excellent efficiency, sensitivity and specificity and could be reliably used for screening and confirmation of ketamine in urine specimens. The data for ELISA and LC-MS/MS analysis of both urine and hair samples found that ketamine and norketamine are present in all specimens and highlighted that ketamine is being abused in Malaysia.

12.2 Further Work

In the study described in Chapter 6, three liquid chromatography columns, 5.00 cm x 4.6 mm, with a frit size of 0.2 µm were packed with S-ketamine MIP, R/S ketamine MIP and NIP. The amount of packing in each column was approximately 0.5 gram polymer. These columns have been pre-evaluated using HPLC-UV and LC-MS/MS. The preliminary data obtained on HPLC-UV analysis using ketamine standards demonstrated the columns can potentially used for LC-MS/MS analysis for direct injection of ketamine and norketamine samples.

Further work should be carried out to investigate whether these columns made in-house based on the MIP materials can successfully be used for the detection of ketamine and norketamine in sample matrices such as urine, pre-extracted blood and hair for direct injections to LC-MS/MS, which has not been tried before. If this was shown to be feasible, it would contribute significantly to forensic toxicology in terms of reducing the analysis time and increasing the selectivity and sensitivity of the method.

To enable this, the columns should be optimised in terms of the column length and diameter, the MIP particle size, column efficiency, column reproducibility, range of pressure applied, and range of pH and solvent to be compatible or better than the commercial polymer based LC columns, especially for LC-MS/MS applications [100]. No studies have yet been published on MIP-based LC-MS/MS columns, even though many preliminary data have been published using HPLC-UV [248, 249].

Also, further work should include studies on the other major metabolite of ketamine, dehydronorketamine, which is now commercially available as a standard, to complete the whole study on ketamine and its main metabolites.

Further work should also be carried out on the chiral selectivity shown by the MIP columns in this preliminary study. This is very important because half of the drugs in use are chiral, including ketamine, and an HPLC column of this type could be used in forensic toxicology to distinguish the active isomer from the less active one [201].

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14 APPENDICES

14.1 Publications in Support of This Thesis

1. Harun N, Anderson RA and Miller EI**. Validation of an Enzyme-Linked Immunosorbent Assay (ELISA) screening method and a Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) confirmation method for the respective identification and quantification of ketamine and norketamine in urine samples from Malaysia**. J Anal. Toxicol. 33. July/August 2009.

2. Norlida Harun and Robert A. Anderson**. Method Optimisation and Validation of the Neogen ELISA Ketamine Kit for Rapid Screening in Forensic Toxicology**. Proceeding of the Naif Arab University for Security Sciences (NAUSS) International Toxicology Conference, Saudi; 5-7 Nov 2007**.**

3. **Harun, N and Anderson RA. Validation of ELISA and LC-MS-MS methods for the determination of ketamine and norketamine in human urine samples**. Proceeding of the 46th The International Association of Forensic Toxicologist (TIAFT), Martinique; 2-9 June 2008.

4. Norlida Harun, Robert A. Anderson, Marinah M. Ariffin, Eleanor I. Miller, Peter A. Cormack. **Potential of molecularly imprinted polymers for extraction of drugs in forensic toxicology: an overview of the Glasgow experience.** Proceeding of The Forensic Science Society Spring Conference: Once Upon A Time There Was A Trace… Nottingham; 24-25 April 2009 and Proceeding 50th Anniversary for Forensic Toxicology in Glasgow University; 26 - 27 March 2009.

5. Norlida Harun, Robert A. Anderson, Peter A.G. Cormack. **Molecularly imprinted solid phase extraction (MISPE) and liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis of ketamine and norketamine in hair samples.** Proceeding of the 42nd IUPAC Congress, Glasgow; 2-7 August 2009.

6. Norlida Harun, Eleanor I. Miller, Robert A. Anderson, Peter A. G. Cormack **Ketamine and norketamine detection in hair by Molecularly Imprinted Solid Phase Extraction (MISPE) versus Solid Phase Extraction (SPE) prior to LC-/MS/MS Analysis**. Proceeding of the 47th The International Association of Forensic Toxicologist (TIAFT),Geneva, Switzerland; 23-27 August 2009.

7. Norlida Harun, Robert A. Anderson and Peter A.G. Cormack. **Analysis of ketamine and norketamine in hair samples using molecularly imprinted solid-phase extraction (MISPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS).** Journal of Analytical and Bioanalytical Chemistry. DOI:10.1007?s00216-009-3404-6. Anal Bioanal Chem (2010) 396:2449–2459.

8. Norlida Harun and Robert A. Anderson. **Application of Molecularly Imprinted Solid Phase Extraction (MISPE) and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) for detection of amphetamines in whole blood.** Accepted for oral presentation of the 48th The International Association of Forensic Toxicologist (TIAFT),Bonn, German; 29 August- 2 September 2010.

Validation of an Enzyme-Linked Immunosorbent Assay (ELISA) screening method and a Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) confirmation method for the respective identification and quantification of ketamine and norketamine in urine samples from Malaysia

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ABSTRACT

An ELISA and a LC-MS/MS confirmation method were developed and validated for the identification and quantitation of ketamine and its major metabolite norketamine in urine samples. The Neogen® ketamine microplate ELISA was optimized with respect to sample and enzyme conjugate volumes and also the sample pre-incubation time before addition of the enzyme conjugate. The ELISA kit was validated to include an assessment of the dose-response curve, intra- and inter-day precision, limit of detection (LOD) and cross-reactivity. The sensitivity and specificity were calculated by comparison to the results from the validated LC-MS/MS confirmation method. An LC-MS/MS method was developed and validated with respect to LOD, LLOQ, linearity, recovery, intra- and inter-day precision and matrix effects. The ELISA dose-response curve was a typical S-shaped binding curve, with a linear portion of the graph observed between 25-500 ng/mL for ketamine. The cross-reactivity of 200 ng/mL norketamine to ketamine was 2.1 % and no cross-reactivity detected with 13 common drugs tested at 10,000 ng/mL. The ELISA LOD was calculated to be 5 ng/mL. Both intra- (n=10) and inter-day precisions (n=50) were below 5.0 % at 25 ng/mL. The LOD for ketamine and norketamine was calculated statistically to be 0.6 ng/mL. The LLOQ values were also calculated statistically and were 1.9 ng/mL and 2.1 ng/mL for ketamine and norketamine. The test linearity was 0-1200 ng/mL with correlation coefficient (R^2) > 0.99 for both analytes. Recoveries at 50 ng/mL, 500 ng/mL and 1000 ng/mL range from 97.9 % to 113.3 %. Intra- and inter-day precision between extracts for n=5 and n=25 respectively for ketamine and norketamine were excellent (< 10 %). Matrix effects analysis showed an average ion suppression of 5.7 % for ketamine and an average ion enhancement of 13.0 % for norketamine for urine samples collected from six individuals. A comparison of ELISA and LC-MS/MS results demonstrated a sensitivity, specificity and efficiency of 100 %. These results indicated that a cut-off value of 25 ng/mL ketamine in the ELISA screen is particularly suitable and reliable for urine testing in a forensic toxicology setting. Furthermore, both ketamine and norketamine were detected in all 34 urine samples collected from individuals socializing in pubs by the Royal Malaysian Police. Ketamine concentrations detected by LC-MS/MS ranged from 22 -31,670 ng/mL and norketamine concentrations ranged from 25 - 10,990 ng/mL. The concentration of ketamine and norketamine detected in the samples are most likely indicative of ketamine abuse.

Keywords: Ketamine, Norketamine, ELISA, LC-MS/MS, Urine

Method Optimisation and Validation of the Neogen ELISA Ketamine Kit for Rapid Screening in Forensic Toxicology

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Introduction: Ketamine is an anesthetic drug used clinically for both humans and animals. A recent increase in ketamine abuse, especially in Far East countries such as Taiwan, Singapore and Malaysia, has led to the development of a growing number of detection methods. Ketamine and its enantiomers have short distribution and elimination half-lives therefore require rapid, sensitive and reliable detection methods. This study optimised and evaluated the suitability of the commercially available Neogen ELISA ketamine kit for use in forensic toxicology.

Materials and Methods: Ketamine ELISA kits were purchased from Neogen Corporation, Lexington, USA. ELISA steps were automated using a Miniprep 75 automated pipettor and the microplates were read at 450 nm with a Sunrise EIA reader from Tecan. Twelve different sets of conditions with respect to sample and enzyme conjugate volumes and incubation times were applied for optimising the method. This was followed by method validation procedures including dose-response curve, intra and inter-day precision, limit of detection, sensitivity, specificity, and cross-reactivity studies. Finally the optimised and evaluated method was tested with human urine samples.

Results and Discussion: The optimised method used 20 µl of sample in each microplate well. No pre-incubation time was required and 180 µl conjugate were added immediately. The plates were then incubated for 45 minutes in the dark, washed with 300 µl diluted phosphate buffer and 150 µl K-Blue substrate was added. The reaction was stopped with 1 N aqueous hydrochloric acid and the plates were read at 450 nm. The test linearity was from 10 ng/ml to 1000 ng/ml and the cross-reactivity for norketamine at 200 ng/ml ketamine was 2.1%. The test showed zero cross-reactivity to amphetamine, methamphetamine, MDA, MDMA, MDEA, cocaine, benzoylecgonine, diazepam, morphine, 6-MAM, methadone, PCP and tiletamine at 10,000 ng/ml. The LOD obtained was 5 ng/ml. Precision was better than 10%, with intraday precision (n=10) 2.47% and inter-day precision (n=50) 4.79% at a concentration of 25 ng/ml. Forty-four urine samples were analysed using the method: 10 control samples from laboratory personnel screened negative and 33 samples from Malaysian Royal Police cases screened positive and one negative at a cut-off of 25 ng/ml. The positive samples had previously been confirmed by GC-MS in the Drug Laboratory, Kuala Lumpur General Hospital, Malaysia. The sensitivity for the test was 97% and the specificity was 100%.

Conclusion: A simple, rapid and efficient ELISA test for ketamine has been optimised and validated. Automated procedures required less then 3 hours for 96-well samples. The test was very specific to the parent compound and showed minimal cross-reactivity to the metabolite norketamine. The precision was good, with a wide linear range dose-response curve. The kit detected ketamine at a range of concentrations in 44 urine samples. It can determine trace concentrations of ketamine and is fit for the purpose of forensic toxicology screening.

Key Words: Ketamine, ELISA, Forensic Toxicology

Validation of ELISA and LC-MS-MS methods for the determination of ketamine and norketamine in human urine samples

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ABSTRACT

Background: A recent increase in the misuse of ketamine as a recreational drug in South East Asian countries such as Taiwan, Singapore and Malaysia has necessitated the development of analytical methods for this drug. In this study, two different techniques were developed for screening and confirmation. **Method:** The commercially available Neogen ELISA kit for ketamine was selected and optimised with respect to sample and enzyme conjugate volumes and the pre-incubation time. Method validation parameters investigated for ELISA were dose-response curve, intra and inter-day precision, LOD, sensitivity, specificity, and cross-reactivity studies. For confirmation, an LC-MS-MS method was developed and validated with respect to LOD, LLOQ, linearity, recovery, precisions and matrix effects. All samples were hydrolysed at 60°C for 3h using ß-glucoronidase from *Helix pomatia*. Ketamine and norketamine were extracted by solid phase extraction using World Wide Monitoring Clean Screen® columns. LC-ESI-MS-MS analysis was carried out using a Thermo Finnigan LCQ Deca XP instrument and chromatographic separation was performed using a Synergi Hydro RP column. Both methods were applied to 34 human urine case samples provided by the Narcotics Department of the Royal Malaysian Police. **Results:** The ELISA test was linear from 25-500 ng/mL. The cross-reactivity for the main metabolite, norketamine at 200 ng/mL ketamine was 2.1% and no cross-reactivity was detected with thirteen other common drugs at a concentration of 10,000 ng/mL. The LOD obtained was 5 ng/mL and the precision was <10%, with intraday precision (n=10) 2.47 % and inter-day precision (n=10 x 5 days) 4.79 % at a cut off concentration of 25 ng/ml. For the LC-MS-MS method, the LODs for ketamine and norketamine were 0.56 and 0.63 ng/mL and the LLOQs were 1.88 and 2.10 ng/mL respectively. The test demonstrated wide linearity over the range of 0-1200 ng/mL with r^2 better than 0.99 for both ketamine and norketamine. The recoveries were acceptable for both analytes at low (50 ng/mL), medium (500 ng/mL) and high (1000 ng/mL) concentrations and ranged from 97.9% to 113.3%. The method demonstrated good intra and inter-day precision (< 10 %). Matrix effects analysis for ketamine showed ion suppression of <10 % while norketamine showed ion enhancement of <20 %. **Conclusion:** A simple, rapid and efficient ELISA screening test for ketamine has been optimised and validated. A complementary sensitive and precise LC-ESI-MS/MS method for confirmation has also been developed and validated. Using the cut off value of 25 ng/mL, the Neogen ELISA demonstrated a very good correlation with the LC-MS-MS method with a sensitivity and specificity of 100%. The LC-MS-MS method detected various concentrations of ketamine and norketamine in 34 human urine samples. Both methods are reliable for routine screening and confirmatory analysis of samples used in workplace drug testing and in forensic toxicology.

Keywords: Ketamine; ELISA; LC-MS-MS, Urine

Potential of molecularly imprinted polymers for extraction of drugs in forensic toxicology: an overview of the Glasgow experience.

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Introduction: A Molecularly imprinted polymer (MIP) is a synthetic polymer bearing on its surface the molecular imprint of a specific target molecule (the template). The polymer has a permanent memory of the template and is capable of selectively rebinding it. To date, the application of MIPs has been dominated by solid phase extraction [1]. The present paper describes the synthesis of MIPs, optimisation of molecularly imprinted solid phase extraction (MISPE) and its application to real hair samples for the determination of drugs prior to LC-MS-MS analysis.

Synthesis: MIPs were prepared by bulk polymerization using ketamine, diazepam and flunitrazepam as templates, using methacrylic acid (MAA) as monomer, ethylene glycol dimethacrylate (EGDMA) as crosslinker and toluene and chloroform as porogens. The templates were extracted from the MIPs with methanol/ acetic acid (9/1, v/v) for 24 h. The monoliths were ground to the desired particle size of 25 to 38 μm and 20 mg of MIP was packed into each SPE cartridges.

Method: MISPE optimisation included template removal, choice of conditioning, washing and elution solvents, studies of binding capacity and cross-reactivity to metabolites and other drugs, and equilibration of the columns with the aqueous environment. After MISPE optimisation, LC-MS/MS methods were validated for each analyte, including measurement of the limit of detection (LOD), lower limit of quantification (LLOQ), linearity, recovery, intra- and inter-assay precision and matrix effects. These methods were applied to hair samples from individuals who had been shown positive for benzodiazepines in post-mortem blood by ELISA and from chronic ketamine users in Malaysia.

Results: Drugs successfully extracted with this application included ketamine and norketamine, diazepam, lorazepam, chlordiazepoxide, temazepam, flunitrazepam, nordiazepam, nitrazepam [2,3]. The ketamine and norketamine levels in chronic ketamine users were quite high, ranged from 0.2-5.7 ng/mg and

0.6 to 1.2 ng/mg. Low levels of benzodiazepines in post mortem hair samples were detected by both diazepam and flunitrazepam as the MIPs templates ranged from 0.02 ng/mg to 1.0 ng/mg.

Conclusion: These studies demonstrated that, in terms of sensitivity, selectivity, lack of interferences and robustness, MISPE can be recommended for accurate and precise determination of drugs in forensic toxicology.

Key words: MIPs, SPE, ketamine, benzodiazepines, hair, LC-MS/MS

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Molecularly imprinted solid phase extraction (MISPE) and liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis of ketamine and norketamine in hair samples

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Summary Text: Molecularly imprinted polymers (MIP) are highly cross-linked polymers synthesised in the presence of template molecules. Post-synthesis, the template is removed, leaving behind imprinted binding sites (cavities) that have a permanent memory of the template and that are capable of rebinding with the template molecule, or structurally similar molecules, in a strong and selective manner. Ketamine is a licenced anaesthetic that is misused as a recreational drug. In this study MISPE and LC-MS/MS were successfully applied to the detection and quantification of ketamine and its metabolite, norketamine, in human hair. The method showed good sensitivity, selectivity, lack of interferences and robustness. MISPE can be recommended for accurate and precise determination of drugs in forensic toxicology

Introduction: Molecularly imprinted solid-phase extraction (MISPE) for the detection of ketamine, an anaesthetic drug which is widely misused as a recreational club drug, in hair has not been reported previously. This study assessed MISPE as an alternative to conventional solid phase extraction for trace detection of drugs in forensic toxicology using ketamine as a model substance.

Synthesis: MIPs were prepared by bulk polymerization using (±)- *ketamine* and (+)-*S-ketamine* as templates, methacrylic acid (MAA) as monomer, ethylene glycol dimethacrylate (EGDMA) as crosslinker and toluene as porogen. Ketamine hydrochloride was first converted to the free base ketamine. Template was extracted from product MIPs with methanol/ acetic acid (9/1, v/v) for 24 h. The monoliths were ground to the desired particle size of 25 - 38 µm. Preliminary evaluation with HPLC-UV in comparison with a non-imprinted polymer (NIP) indicated that both (±)- *ketamine* and (+)-*S-ketamine* had been imprinted. 20 mg of MIP was then packed into each SPE cartridge for the study.

Method: Optimisation of MISPE included template removal, selection of solvents use in the extraction and studies of binding capacity and cross-reactivity to

metabolites and other drugs. After MISPE optimisation, LC-MS/MS methods were validated for each analyte, including measurement of the limit of detection (LOD), lower limit of quantification (LLOQ), linearity, recovery, intra- and inter-assay precision and matrix effects. These methods were applied to hair samples from chronic ketamine users in Malaysia.

Results: Template was removed by sequential treatment with acetonitrile, chlororm and 30 % methanol in acetic acid. Recovery of ketamine was 87 % for (±)-*ketamine* using the MIP compared to 32 % using the NIP. The binding capacity was 0.125 µg/mg polymer, which was sufficient for the analytical application. LODs for ketamine and norketamine were 0.10 and 0.14 ng/mg hair and LLOQs were 0.37 and 0.47 ng/mg hair, respectively. The method was linear from 0-10 ng/ mg hair with linear correlation coefficients (R^2) better than 0.99 for both ketamine and norketamine. The recoveries from adulterated hair samples were 86 % for ketamine and 88 % for norketamine at concentrations of 50 ng/mg, The method demonstrated good intra- and inter-day precision of <5% based on analysis of pooled hair samples for both analytes. Minimal matrix effects were observed during LC-MS/MS analysis of ketamine (ion suppression, -6.8 %) and norketamine (ion enhancement + 0.2 %). Ketamine and norketamine concentrations in the samples analysed were relatively high, ranging from 0.2-5.7 ng/mg and 0.6 to 1.2 ng/mg, indicating that the samples were from chronic ketamine users.

Conclusion: This study demonstrated that MISPE combined with LC-MS/MS can provide a sensitive, selective, robust and clean method of analysis which can be recommended for accurate and precise determination of drugs in forensic toxicology.

Key words: MIPs, MISPE, ketamine, hair, LC-MS/MS

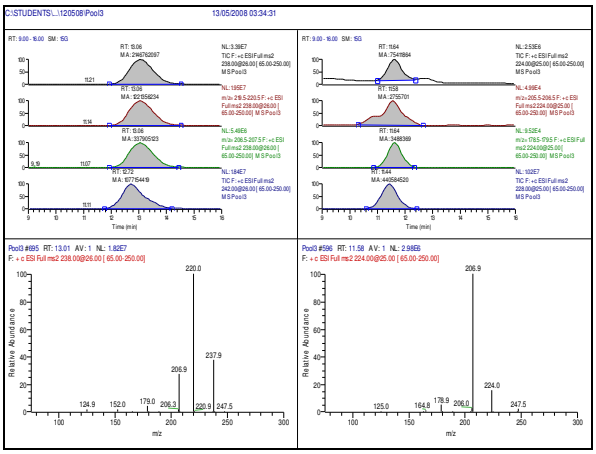
Graphic Abstacts for IUPAC Conference

Title: Molecularly imprinted solid phase extraction (MISPE) and liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis of ketamine and norketamine in hair samples

Authors: Norlida Harun#, Peter A. Cormack and Robert A. Anderson

Summary Text: In this study MISPE and LC-MS/MS were successfully applied to the detection and quantification of ketamine and norketamine in hair. The method showed good sensitivity, selectivity, lack of interferences and robustness. MISPE can be recommended for accurate and precise determination of drugs in forensic toxicology

Key words: MIPs, MISPE, ketamine, hair, LC-MS/MS



LC-MS/MS analysis of ketamine and norketamine in an extract of pooled human hair from ketamine abusers.

Ketamine and norketamine detection in hair by Molecularly Imprinted Solid Phase Extraction (MISPE) versus Solid Phase Extraction (SPE) prior to LC-/MS/MS analysis

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Background: This preliminary study was a comparison of validated MISPE and conversional SPE methods coupled to LC-MS/MS analysis in hair samples from living subjects. An anti-ketamine imprinted polymer was synthesized and used as MISPE sorbent; the polymer was capable of rebinding ketamine or structurally similar molecules, in a strong and selective manner.

Samples: Blank hair samples for method development and validation were from volunteers in the laboratory. The method application was tested on real hair samples collected from four chronic females' ketamine users at a drug prevention centre in Malaysia.

Method: Hair samples were decontaminated with 0.1% aqueous sodium dodecyl sulfate (SDS), deionised water and dichloromethane, then air dried. 10 ± 0.1 mg samples plus 50 ng ketamine and norketamine internal standards were incubated for 18 hours at 45 ºC in 1.5 mL 0.1 M phosphate buffer pH 5.0 and subsequently extracted by SPE and MISPE followed by LC-MS/MS analysis. Clean Screen® (ZSDAU020) cartridges were used for SPE and compared with the MISPE.

Results: MISPE and SPE coupled with LC-MS/MS methods were linear from 0-10 ng/ mg hair with R^2 better than 0.99 for both ketamine and norketamine. For MISPE, the LODs for ketamine and norketamine were 0.10 ng/mg hair and LLOQs were 0.4 and 0.5 ng/mg hair while for SPE, LODs were 0.5 ng/mg for ketamine and norketamine and LLOQs were 0.9 and 1.8 ng/mg. The recoveries were above 85% for both analytes and methods. The average ketamine and norketamine intra- and inter-batch imprecision were <5% for the pooled hair sample on both methods. MISPE showed low matrix effects in hair during LC-MS/MS analysis; ketamine (ion suppression, -6.8 %) and norketamine (ion enhancement + 0.2 %) whereas very significant values was demonstrated in SPE method for the two analytes. MISPE successfully detected ketamine and norketamine in all the four samples. The ketamine and norketamine levels ranging from 0.2-5.7 ng/mg and norketamine from <0.5 ng/mg to 1.2 ng/mg. SPE detected ketamine ranging

from 0.5 – 6.7 ng/mg but norketamine were not detected in 2 out of the four samples and less than LLOQ in the other 2 samples.

Conclusion: The MISPE coupled with LC-MS/MS demonstrated good selectivity and sensitivity for ketamine and norketamine in hair. SPE coupled with LC-MS/MS showed higher selectivity to ketamine than MISPE but less sensitivity for norketamine. High matrix effect for SPE method might interfere with the LC-MS/MS analysis and caused less sensitivity for norketamine detection in hair.

Key words: MIPs, SPE, ketamine, norketamine, hair, LC-MS/MS

Molecularly imprinted solid-phase extraction (MISPE) and liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis for ketamine and norketamine determination in hair samples

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Abstract

An anti-ketamine molecularly imprinted polymer (MIP) was synthesized and used as the sorbent in a solid-phase extraction protocol to isolate ketamine and norketamine from human hair extracts prior to LC-MS/MS analysis. Under optimised conditions, the MIP was capable of selectively rebinding ketamine, a licensed anaesthetic that is widely misused as a recreational drug, with apparent binding capacity of 0.13 µg ketamine per mg polymer. The limit of detection (LOD) and lower limit of quantification (LLOQ) for both ketamine and norketamine were 0.1 ng/mg hair and 0.2 ng/mg hair, respectively, when 10 mg hair was analysed. The method was linear from 0.1-10 ng/mg hair, with correlation coefficients (R^2) better than 0.99 for both ketamine and norketamine. Recoveries from hair samples spiked with ketamine and norketamine at a concentration of 50 ng/mg were 86% and 88% respectively. The method showed good intra- and inter-day precision (<5%) for both analytes. Minimal matrix effects were observed during LC-MS/MS analysis of ketamine (ion suppression -6.8%) and norketamine (ion enhancement +0.2%). Results for forensic case samples demonstrated that the method successfully detected ketamine and norketamine concentrations in hair samples with analyte concentrations ranging from 0.2-5.7 ng/mg and 0.1 to 1.2 ng/mg, respectively.

Keywords: MIPs, SPE, ketamine, norketamine, hair, LC-MS/MS

Application of Molecularly Imprinted Solid Phase Extraction (MISPE) and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) for detection of amphetamines in whole blood

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Background: A commercial molecularly imprinting based column (Amphetamines SupelMIP ™) from Supelco was used in extraction prior to LC-MS/MS analysis for detection of five types of amphetamines in post mortem whole blood. The columns were previously evaluated by Supelco for use of amphetamines detection in urine and demonstrated high recoveries. Seven case blood samples were obtained from Forensic Medicine and Science, University of Glasgow. The LC-MS/MS method underwent method validation procedures and the results were compared with the previously obtained results of the routine GC-MS procedures.

Method: *Pre-treatment:* Internal standard was spiked into 1.0 ml aliquots of bloods which were diluted with 1.0 ml 10 mM ammonium acetate buffer pH 8.0, vortexed mixed and centrifuged at 3000 rpm for 10 minutes. *Extraction:* The column was conditioned with 1.0 ml methanol, followed by 1.0 ml 10 mM ammonium acetate buffer, pH 8.0 without application of a vacuum. The pre-treated sample was loaded on the SPE cartridges then cartridges were washed sequentially with 2 x 1.0 ml DI water, not to let the column dry out, 1.0 mL 60/40 v/v MeCN/DI water followed by a drying step of 5-10 minutes with full vacuum, and finally with 1.0 mL of 1 % HOAc in MeCN. 2 x 1.0 mL 1 % formic acid in MeCN was used to elute the amphetamines with mild application of vacuum between each elution. The SPE eluant was evaporated to dryness under a stream of nitrogen gas without heating. The residues were reconstituted with 100 µL initial mobile phase and vortex mixed prior to LC-MS/MS analysis.

Results: MISPE LC-MS/MS methods were linear from 0-1000 ng/ml blood with R^2 better than 0.99 for the five amphetamines. The LODs were 0.2 – 0.6 ng/ml and the LLOQ were from 0.8 -1.9 ng/ml for all 5 amphetamines. Matrix effects were within ± 10 for 3 levels (50, 450 and 900 ng/ml) for the 5 amphetamines. The recoveries were between 32.4 to 61.4 with RSD less than 11 % compared to 97 to 113 with RSD of 1.4 to 9.8 for the same group analysed in urine. The intra and inter-day precision RSD were less than 10 %. The method detected the same class of amphetamines in all samples but the concentrations were lower than comparison GC-MS method but detected low levels of amphetamines compared to comparison GC-MS.

Conclusion: The method demonstrated good linearity and precision with low matrix effects. The LOD and LLOQ were better than the comparison GC-MS method and suitable for detection of low levels of amphetamines in post mortem blood. The case samples results were lower than the result obtained by the routine GC-MS may be resulted from incomplete protein precipitation step ahead of extraction and also may be affected by gap of reanalysed (6 months to one year) which some degradation may occurred in the whole blood especially for MDMA and MDEA.

Key words: MISPE, amphetamines, whole blood, LC-MS/MS

14.2 Published Papers