Detection of ketamine in human plasma by High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC)- A Preliminary Study

by

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LIST OF ABBREVIATIONS AND SYMBOLS

HPLC	High Performance Liquid Chromatography
TLC	Thin Layer Chromatography
LLE	Liquid-liquid extraction
SPE	Solid phase extraction
cm	Centimetre
mm	Millimetre
µm	Micrometre
µl	Microliter
µg/ml	Microgram per millilitre
I.D	Internal diameter
ml/min	Millilitre per minute
mg/ml	Milligram per millilitre
UV	Ultra violet
nm	Nanometre
rpm	Rotation per minutes
ºC	Temperature or Celsius scale
g	Gram
min	Minutes
µV*sec	Microvolt-seconds
pH	Negative logarithm of hydrogen ion concentration

ABSTRAK

Ketamine ialah salah satu bahan psikoaktif baru (NPS) dan penagihan dadah ini semakin meningkat. Tujuan kajian ini ialah untuk mengesan ketamine dalam plasma manusia menggunakan dua teknik kromatografi yang berbeza iaitu kromatografi cecair berprestasi tinggi (HPLC) dan kromatografi lapisan nipis (TLC). Pengekstrakan cecair-cecair (LLE) digunakan untuk mengekstrak ketamine dari plasma. Dalam analisis HPLC, fasa pegun yang digunakan ialah kolum C18 (150 X 4.6 mm, saiz zarah 2.7 µm) manakala fasa gerak ialah 23% asetonitril dan 77% larutan tampan fosfat dengan pH 7.2. Pengesan yang digunakan dalam analisis HPLC adalah melalui pengesan fotodiod pelbagai (PDA). Pengoptimuman kromatografi dilakukan dengan menggunakan kadar aliran yang berbeza dan juga kepekatan ketamine yang berbeza. Kadar aliran 1.0 ml/min telah dipilih dan kepekatan paling rendah yang boleh dikesan menggunakan 'HPLC Waters 2695 Separation Module' ialah 5 µg/ml. Pengoptimuman ekstraksi dilakukan dengan menggunakan pengekstraksi yang berbeza. Peratus perolehan untuk kedua-dua bahan pelarut ialah lebih daripada 100%. Dalam analisis TLC untuk ketamine dalam plasma manusia, fasa pegun ialah HPTLC Silica gel 60 F_{254}. Teknik ekstraksi yang berbeza digunakan dalam analisis ini. Ekstraksi 'Acid-back' dan fasa gerak yang mengandungi etil asetat: metanol: ammonia pekat 28% (8.5: 1:0.5) memberikan pemisahan yang baik untuk ketamine. Nilai Rf untuk ketamine ialah 0.92. Kepekatan paling rendah untuk mengesan ketamine dalam plasma manusia menggunakan teknik TLC ialah 10 µg/ml. Dalam kajian ini, ketamine boleh dikesan menggunakan teknik TLC dan HPLC. Pengoptimuman pengekstrakan cecair-cecair untuk analisis HPLC diperlukan dalam kajian masa depan .

ABSTRACT

Ketamine was one of the new psychoactive substance (NPS) which is increasingly abused nowadays. The aim of this study was to detect the ketamine in human plasma by two different chromatographic methods of high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Liquid-liquid extraction (LLE) for ketamine in human plasma was used for both methods. In HPLC analysis of ketamine, the stationary phase for HPLC separation of ketamine and internal standard, morphine in human plasma was C18 column (150 X 4.6 mm I. D, particle size 2.7 µm) and the mobile phase consist of 23% acetonitrile and 77% phosphate buffer with pH 7.2. The HPLC system was equipped with photodiode array (PDA) detector. The optimization of chromatographic conditions was done by varying the flow rate of mobile phase and different concentration of drug standard. Flow rate 1.0 ml/min was chosen and the lowest concentration of ketamine that can be detected by the HPLC Waters 2695 Separation Module was 5 µg/ml. The optimization of extraction was done by varying the types of solvents used for extraction of drug from plasma. The percentage recoveries obtained for the solvents used in this study was more than 100%. In TLC analysis of ketamine in human plasma, the stationary phase was HPTLC Silica gel 60 F_{254}. The analysis was done by varying the liquid-liquid extraction methods and varying the mobile phase. The good separation of ketamine was achieved using acid-back liquid extraction method and mobile phase consist of ethyl acetate: methanol: concentrated ammonia solution 28% (8.5:1:0.5). The Rf value calculated was 0.92 and the lowest concentration for ketamine in plasma that can be detected was 10 µg/ml. Ketamine was detectable with TLC and HPLC methods. The optimization of liquid-liquid extraction for HPLC analysis was required in future studies.

CHAPTER 1: INTRODUCTION

1.1 Study background

Ketamine or ketamine hydrochloride was first introduced as an anaesthetic in 1964 (Morgan and Curran, 2011). The historical used of this drug begin when it becomes the major anaesthetic for American soldiers injured during the Vietnam War (Morgan and Curran, 2011). Ketamine also broadly used in Veterinary Medicine with purpose of inducing anaesthesia in almost all kind of animals such as birds, primates, and also exotic or wild animals (Sori *et al.*, 2013). Since ketamine was universally used in human and also animals, it comes out with several trade name and also street name. The trade names for ketamine include Ketalar, Ketaject (Phoenix Pharmaceutical, Burlingame, CA), Ketaset, and Vetalar. The synonyms for ketamine on the street or the street names are K, Kit - Kat, Kay, Jet, Vitamin K, Special K, Super Acid, Super K, Super C, Special LA Coke, and 1980 Acid (Barceloux, 2012).

This anaesthetic drug has ability of causing delusions, hallucination, confusion, and sometimes 'out-of-body' and 'near-death' experience (Jansen, 1991). Therefore, ketamine has been wrongly used as a drug of abuse in most drug addicts. Route of admission for ketamine is normally through smoking in which ketamine is added onto tobacco or cannabis leaves (Barceloux, 2012). In addition, ketamine is difficult to manufacture in clandestine laboratory, therefore the illicit ketamine comes from a pharmaceutic preparation diverted from a legal stock especially from veterinarian clinics or pharmaceutical laboratory.

In forensic toxicology analysis, detection for drug of abuse in biological matrix is inevitable. Ketamine analysis from biological matrix commonly collects urine sample because sampling is easy, less invasive and has prolonged detection of substance (Ščavničar *et al.*, 2015). However, dilute and contamination during sampling of such sample are potential issues and sampling method may intrude one's privacy (Yew,

2015). On the other hand, human plasma is known to have less contaminants, gives improved detection and able to provide higher ketamine concentration (Jemal *et al.*, 2003) despite the intrusive nature in obtaining blood sample from the subject.

1.2 Problem Statement

Increasing abuse of ketamine as club drugs has been reported around the globe and also at national level (Brown and Melton, 2010; Chemi et al., 2014). The use of ketamine was reported in several countries in South Amerika and Asia. Experts from East and South-East Asia have been reported that ketamine was used in high level in Asia's country which including Brunei Darussalam, China, Indonesia, Singapore, and also Malaysia (United Nations Office on Drug and Crime (UNODC) World Drug Report, 2013). Thus, the increasing use of ketamine cause the raise in analysis of this drug in most laboratory.

Routinely, the analysis of ketamine was performed in urine sample. However, it sometimes can be problematic due to dilute ketamine concentration in urine that can hamper detection of the drug (Jemal et al., 2003). Thus, in this study plasma as a biological matrix will be tested to see if it can improve detection of ketamine. For instrumental analysis, ketamine usually analyse by gas chromatography but this method require derivatization. It is not suitable for a fast detection test of ketamine in human plasma. Instead, liquid chromatography has become the method of choice for determination of this club drug in human plasma as it a simple, sensitive and straightforward method. The time consumption also can be reduced during extraction process as it do not require derivatization.

Generally, the extraction process employed before liquid chromatographic analysis is solid phase extraction (SPE). SPE is one of extraction of choice but it is expensive and not affordable for a common laboratory. Liquid-liquid extraction will be suitable method

for extraction because it is cheaper and easily to handle. In Toxicology Laboratory at Pharmacology Department, School of Medical Science, the TLC of drug was only applied using urine as biological matrix. Therefore, this work attempts to optimise the simple existing extraction and liquid chromatography methods of TLC and HPLC using plasma samples to improve detection of ketamine drugs.

1.3 Objective of study

1.3.1 General Objective

To detect the presence of ketamine in human plasma by two chromatographic techniques, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

1.3.2 Specific Objective

- To apply the liquid-liquid extraction (LLE) techniques for extraction of ketamine in human plasma.
- To test whether TLC method used to detect ketamine in urine is applicable with human plasma.
- To optimize the detection of ketamine in plasma by high performance liquid chromatography(HPLC)

1.4 Research Question

- ❖ TLC method detection of ketamine in urine is applicable or not for detection of ketamine in plasma?
- ❖ Could ketamine in plasma be detected using simple established HPLC analysis from Bolze & Boulieu, 1998 by HPLC system available in USM's Toxicology Laboratory?

1.5 Significance of study

The significance of this study is to improve detection of ketamine from human plasma through rapid and simple preparation of liquid chromatographic analysis (TLC and HPLC). For TLC analysis, the existing method for detecting ketamine in urine was obtained from the Toxicology Laboratory and it was applied and tested whether it can be used for the ketamine drug separation in plasma. For HPLC analysis, the optimum drug separation will be achieved by optimizing the existing method from Bolze and Boulieu, 1998 using the HPLC system available in laboratory. This will give benefit for the researchers and for the laboratory itself to detect the presence of ketamine drug in human biological matrix (i: e plasma).

The use of plasma as biological matrix also gives significance in term of low contaminant during analysis and also able to detect higher concentration of ketamine (Jemal *et al.*, 2003). Thus, it will be a simple and sensitive method for analysis. Besides, extraction of ketamine from human plasma by liquid-liquid extraction (LLE) is cheaper as compared to the commonly used solid-phase extraction (SPE) techniques.

CHAPTER 2: LITERATURE REVIEW

2.1 Ketamine

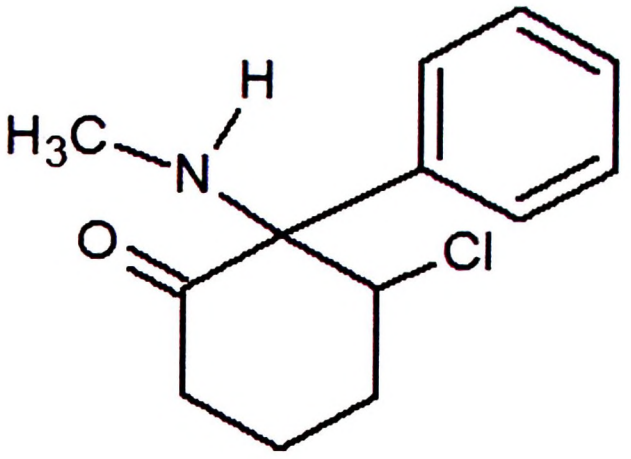
Ketamine is classified by UNODC as a new psychoactive substance (NPS) (*United Nations Office on Drug and Crime World Drug Report*, 2013). NPS means the psychoactive substance in the 'pure form' or in 'preparative form' that is not under international control but can cause the health threat. The effect is similar with the controlled substance under 1961 Single Convention on Narcotic Drugs and 1971 Convention on Psychotropic Substance. The term 'new' means the old substance that is currently raise in marketing and it is not the new drug invention since ketamine was produced in early 1960s.

2.1.1 Physical and Chemical properties of ketamine

Ketamine is an arycycloalkylamine compound, 2(2-chlorophenyl) - 2 - (methylamino) – cyclohexane (Figure 2.1a) with molecular formula $C_{13}H_{16}ClNO$ and molecular weight of 237.72524 g/mol. It is a basic drug with pKa of 7.5 and moderately polar in nature. The class of organic compound for ketamine is chlorobenzenes due to the attachment of one or more chlorine atoms to a benzene group.

Chemically, ketamine is closely related to the internationally controlled drug phencyclidine (PCP) or also known as 'angel dust' (Figure 2.2b). Ketamine is one of the derivatives of PCP, but it is less potent as compared to the PCP (Koek *et al.*, 1989). Previously, the intravenous anaesthetic applied in medical treatment is PCP in 1950s. However, because of the undesired hallucinogenic and delirium effect of PCP, it has been withdrawn and replaced with ketamine as a medical alternative to PCP (United Nation Office on Drugs and Crime, 2016).

a)



b)

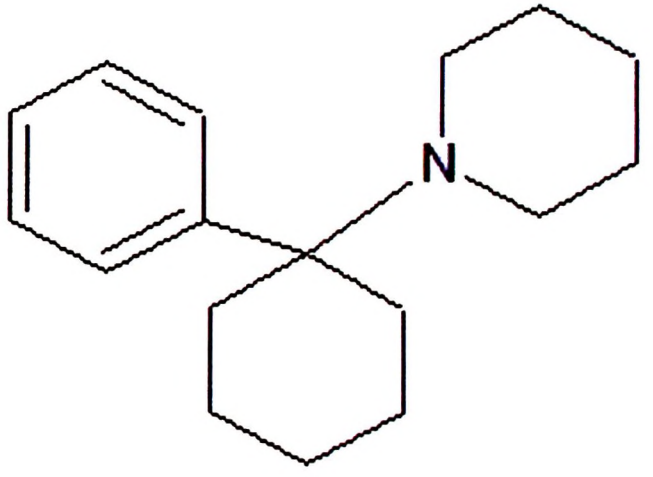


Figure 2.1: a) The chemical structure of ketamine

b) The chemical structure of phencyclidine

2.1.2 Mechanism of action

The mechanism of action for ketamine is primarily by non-competitive antagonism of the N-methyl D-aspartic acid (NMDA) receptor. It acts on the central nervous system (CNS) and has local anaesthetic properties (Pai and Heining, 2007). Besides, it also interacts with opioid receptors, monoamine, cholinergic, purinergic and adrenoceptor system (Kurdi *et al.*, 2014). However, the affinity of ketamine for these receptors is ten times lower than for the NMDA channel. The prove of antagonist interaction of ketamine with monoaminergic, muscarinic, and nicotinic receptors also found as it produce the anticholinergic symptoms such as tachycardia and bronchodilation (Pai and Heining, 2007).

2.1.3 Ketamine abuse

The increasing abuse of ketamine as club drugs make it as one of drug-facilitated sexual assault or also called the 'date rape drug' (Bokor and Anderson, 2014). Relatively, it has been consumed to enhance the sexual experience (Lim, 2003). According to Brown and Melton (2010), ketamine was frequently consumed by young adults and they reported that the club drug use has strong relative with criminal activities and alcohol dependence (Wu *et al.*, 2006). Due to its dissociative anaesthetic, it becomes the preferred drug of abuse and lead to addiction. The methods of administration for ketamine includes intravenous, intramuscular, snorting, and adding to smoking material (Bokor and Anderson, 2014).

2.1.4 Analysis of ketamine

The analysis of ketamine in forensic toxicology usually performed by using the available methods in common laboratory for example immunoassays and chromatographic analysis. Ketamine usually analyse in the biological matrix. Common

biological matrix collected for toxicology analysis are urine, blood, plasma, hair, sweat, saliva, fingernail, bone, serum, stomach content, brain, bile, bladder, liver and many more (Brown and Melton, 2010; Mali *et al.*, 2011).

In this study, plasma sample was chosen and few studies had been reported in detecting ketamine in plasma which includes gas chromatography mass spectrometry (GC-MS) (Liana *et al.*, 2012), secondary ion mass spectrometry (Hua-YangLiao *et al.*, 2015), liquid chromatography (LC) (Aboul-Enein and Hefnawy, 2005), and high performance liquid chromatography(HPLC) (Gross *et al.*, 1999; Sori *et al.*, 2013; Svensson and Gustafsson, 1996; Yanagiharaa *et al.*, 2000).

2.2 Principle of Chromatography

Chromatography is an analytical techniques that is widely used in many field with purpose of separating the specific compound in a mixture. It is a general terminology which applied to the various separation techniques based on the partitioning or distribution of a sample (solute) between a moving or mobile phase and a fixed or stationary phase (Ismail, 2010). All chromatographic techniques consists of mobile phase and stationary phase. Each phase works together to separate the molecule of interest. Mobile phase functioning as the transport medium for analyte whereas stationary phase allows the interaction of the analyte according to its chemical structure and gives the results of separation. There are a lot of chromatographic techniques which applied worldwide including thin layer chromatography, gas chromatography, paper chromatography, ion-exchange chromatography, affinity chromatography, and high performance liquid chromatography.

Generally, two phase involves in chromatographic analysis which are normal phase and reverse phase. Normal phase or liquid adsorption chromatography composed of polar stationary phase and nonpolar mobile phase. The migration or separation of the

analyte in normal phase chromatography is based on the polarity. The less polar analyte, the faster the elution time.

For the reverse phase chromatography, it is opposite to the normal phase chromatography in which the stationary phase is nonpolar and the mobile phase is polar means that the less polar analyte will separate in longer elution time. This phase is the most commonly used in research field and also the most known technique for separation of the substance since it can be applied to a wide range of molecules such as charged and polar molecules. It also provide the precise control of variables for example the organic solvents type, concentration, pH, and temperature. Reverse phase usually used in the advanced chromatographic techniques such as high performance liquid chromatography.

2.3 Liquid- liquid extraction (LLE)

In toxicological analysis, especially in forensic field, the concentration of drugs might be very low in the biological sample. Hence, sample preparation is compulsory to obtain a clean extract and maximum concentration of drug in the biological matrix. There are two types of extraction available which are liquid-liquid extraction (LLE) and solid phase extraction (SPE). Liquid-liquid extraction is the traditional method that is applied in most laboratory as it is cheaper and the materials required is available. The solvent chosen for LLE is important and the pH also must be considered. pH of aqueous phase should be adjusted according to the neutral form of drugs but not become hydrolysed (Pauline, 2000). For the extraction of ketamine from biological fluid, few studies had been conducted by liquid-liquid extraction method (Bolze and Boulieu, 1998), (Yanagiharaa *et al.*, 2000), (Gross *et al.*, 1999), (Li *et al.*, 2012). These studies shows that LLE is an affordable and simple techniques for the sample preparation of ketamine.

2.4 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is one of the most useful technique used as it is a simple, sensitive and quick separation of the specific substance. It compose of stationary phase and mobile phase which available in all the chromatographic technique. HPLC is also called high pressure liquid chromatography due to the need of a high pressure to force the mobile phase through the column or the stationary phase.

Generally, the study of substance by HPLC involving method validation, optimization, and development. HPLC is suitable for analysis of polar, ionic and thermally unstable materials as not limited like GC in the appropriateness by component volatility or thermal stability.

2.4.1 HPLC instrumentation

Components in HPLC system compose of solvent reservoir, pump, degasser, injector, column, and detector. Figure 2.3 shows the components in the HPLC system.

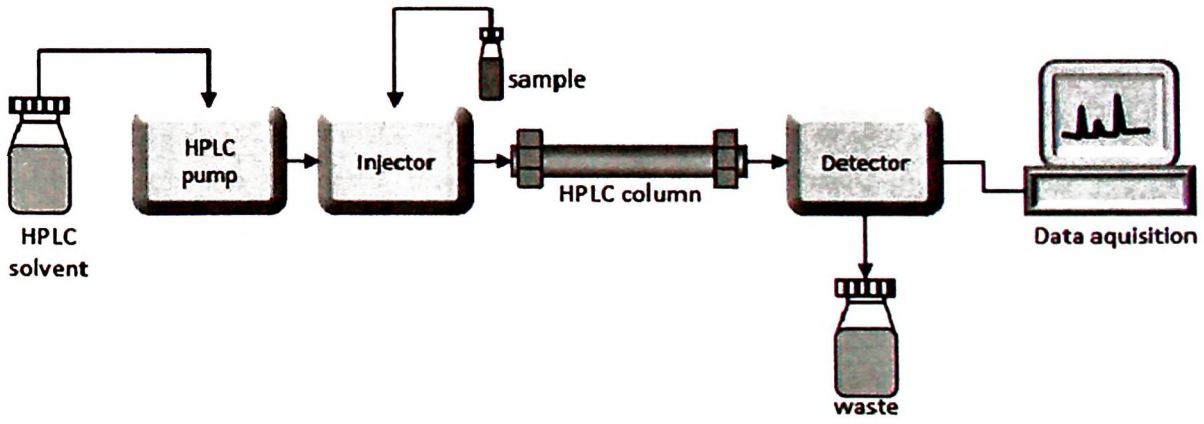


Figure 2.2: The components in HPLC system (Czaplicki, 2013).

2.4.1.1 Solvents

Solvents or mobile phase in HPLC could be neat liquid such as water, acetonitrile or methanol. It also could be mixtures of those liquid, and the liquid with modifiers for example buffer solution. All the solvents must be in a HPLC grade, have a high spectroscopic purity and dust-free. It is necessary to filter and degassed before use.

The mobile phase for HPLC must be certainly be chosen for its chromatographic properties. It is essential to collaborate with a suitable stationary phase for a fast and efficient separation of substance. Normally, the optimization of the chromatographic conditions were made by changing the different types of mobile phase, the percentage, and also the pH of the mobile phase. For analysis of ketamine, the mobile phase that commonly used are the mixture of a buffer solution with the organic components.

2.4.1.2 Pumps

There are several types of pumps which can be used in HPLC. There are three known types of pumps that are reciprocating pump, syringe pump or displacement pump, and pneumatic pump (Robards *et al.*, 2004). Reciprocating pump is a motor driven reciprocating piston which control the flow of the mobile phase. Syringe pump provides the constant flow and lastly, the pneumatic pump delivers the constant pressure. The most commonly used pump in analytical HPLC is reciprocating pump as it has the ability to achieve a wide range of flow rates. This pump is also significantly smoother (Robards *et al.*, 2004).

There are some criteria for a suitable pump which are user friendly, precise and accurate flow rate deliver which contribute to detector noise, allow rapid solvent change. The materials of construction must inert towards the solvent used (Pauline, 2000).

2.4.1.3 Injector

Basically, there are two types of injectors currently used in HPLC which are manual injector and auto-injector. Both injectors provide the precise volume of sample and consistently deliver the sample into the flow path of the HPLC system.

2.4.1.4 Column

Column or stationary phase should be suitable to be used for HPLC analysis. Few types of column typically applied that are C18, C8, and RP-18. The HPLC column must resistant to the usual HPLC pressure, relatively inert to chemical corrosion, no rough surface, grooves, or microporous structure inside the column (Meyer, 2004). There are various size of column which available for the chromatographic analysis, different size and diameter of the column has its different functions which usually for the analytical and preparative purpose. Normally, the column with 10-25 cm long and 2.1-4.6 mm I.D were applied in HPLC. The columns are made of stainless steel to cope with the high backpressure. The column must also glass lined to avoid metal catalysis of solvent-solute reactions (Pauline, 2000).

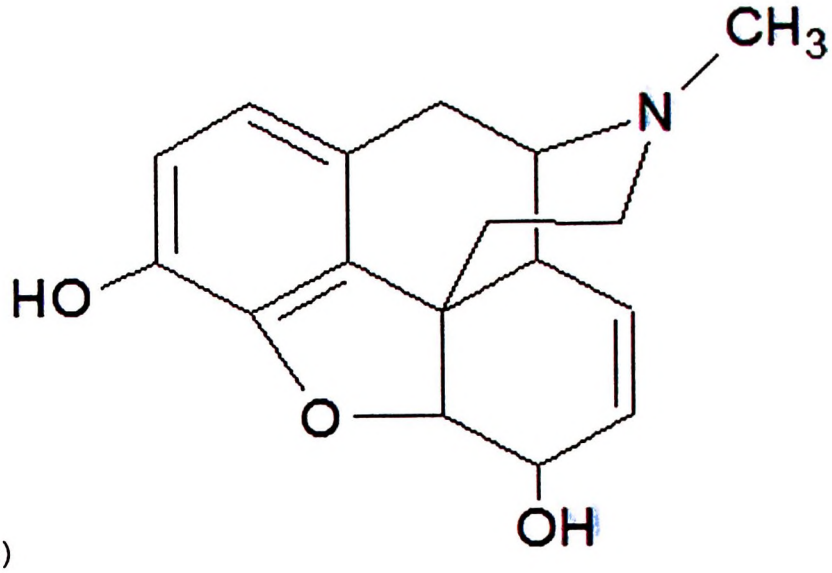
2.4.1.5 Detectors

Detector is the final components in the HPLC system. There are a broad range of detectors currently applied for example UV-vis, refractive index, and fluorescence detector. UV-vis detectors has two basic forms which are fixed-wavelength and photodiode-array (PDA). The suitable detectors must be a stable which mean low rift and noise level, high sensitivity, wide dynamic linear range, fast response, non-destructivity, not sensitive to the changes in mobile phase flow rate, mobile phase

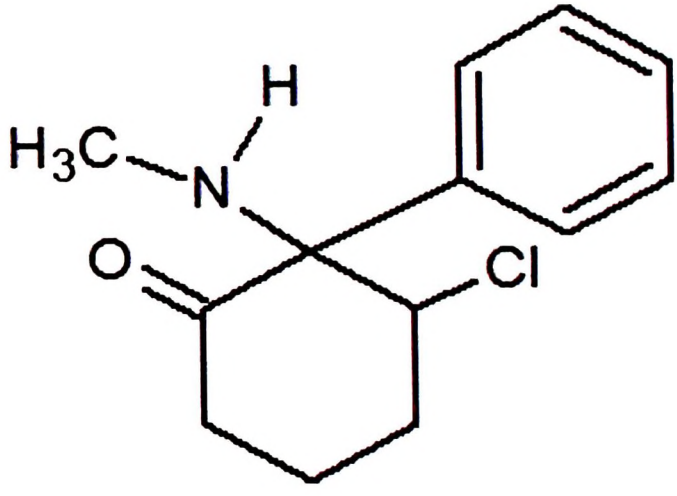
composition, and also changes in temperature and pressure (Dong, 2006b; Pauline, 2000; Po and Irwin, 1980; Swartz, 2010).

2.4.2 Internal standard

The internal standard is necessary in HPLC analysis. It must be pure, clearly defined compound, and have the similar properties with respect to sample preparation. In this study, morphine with molecular formula of $C_{17}H_{19}NO_3$ and molecular weight of 285.33766 g/mol was chosen as the internal standard as it has the similar properties to the ketamine. Morphine is a basic drug with pKa 8.21 and pH 8.5 which is suitable as the internal standard for ketamine. Figure 2.3 shows the chemical structure of morphine and ketamine.



a)



b)

Figure 2.3 : a) The chemical structure of morphine

b) The chemical structure of ketamine

2.4.3 Optimization in HPLC analysis

The optimization in HPLC is achieved in many ways, it depends on how many the variables that is adjusted. It could be the optimization of chromatographic conditions and also the extraction. The optimization in HPLC analysis considered a few variables such as pressure, temperature, mobile phase, flow rate, and pH. The importance in optimization was to ensure that the peak shape, peak resolution was suitable and preventing the baseline noise in chromatogram also necessary.

2.4.4 Optimization of extraction method

For liquid-liquid extraction of a compound, the use of different extraction solvent was necessary to identify whether the drug to be analysed was suitable with those extraction solvent. The calculation of percent recovery was made by calculating the peak area of internal standard and the sample.

2.5 Thin layer Chromatography (TLC)

The term 'thin layer chromatography' was first introduced by E. Stahl in 1956 which means the chromatographic separation process where the stationary phase compose of a thin layer applied to a solid substrate or "support" (Hahn-Deinstrop, 2000). TLC is one of chromatographic techniques that is used for decades by researchers and chemists in many fields including pharmaceuticals, food, and environmental analysis. This is because of the low cost, use of simple and extensively available laboratory equipment, easily to handle, and also able to run many samples in one time (Bernard-Savarya and Pooleb, 2015).

Thin layer chromatography (TLC) is generally performed on the samples as screening test prior to confirmatory analysis using either liquid or gas chromatography. This

technique consist of mobile phase and stationary phase. The mobile phase applied in TLC could be one type of solvent or the mixture of solvents. The example of mobile phase that can be used are methanol, ethyl acetate, and mixture of methanol and ethyl acetate. Mobile phase acts as the developing liquid which carrying the compound and moves throughout the stationary phase by capillary action.

The stationary phase in TLC composed of many types which depends on the analyte. Silica gel, aluminium oxide, cellulose, and magnesium silicate are among the examples of the stationary phase or adsorbent material. The most commonly adsorbent for TLC of ketamine is silica gel (Khajeamiri *et al.*, 2011; Sams and Pizzo, 1987). The TLC adsorbent material is made of finely ground matrix that coated on thin layer of glass plate, metal or plastic film.

In TLC, the separation of compounds were based on its polarity. Migration of more polar compound will be slower than the less polar compound, the less polar compounds migrates first from the polar stationary phase based on the concept of 'like dissolve like' which means the polar compounds stick to the polar stationary phase and thus, it moves slower. For the analysis of compounds by TLC, it require five basic steps which are preparation of developing chamber, preparation of TLC plate, spot the TLC plate, and visualisation.

The analysis by TLC was quantify by calculating the retardation factor or also called Rf value which is defined as the value obtained by dividing the distance travelled by the substance with the solvent front. The Rf value also useful for identification of unknown substance as it is quite specific to different substance provided that reference substance is analysed on the same TLC plate. The formula was as follows:

$$Rf = \frac{distance\ travelled\ by\ substance}{solvent\ front}$$

CHAPTER 3: METHODOLOGY

3.1 Research Design

This study involves the experimental study of ketamine in human plasma by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The source of human plasma was from personal investigator's blood. It was approved by ethics under code (USM/JEPEM/16030091). The documents required and letter for the approval were shown in Appendix C, the Gantt chart in Appendix D, and flow chart in Appendix E. The letters from JEPeM for this research project were attached in Appendix A, B, and F.

3.2 Location of study

This study was fully conducted in Toxicology Laboratory under Pharmacology Department, School of Medical Science, Universiti Sains Malaysia (USM).

3.3 High Performance Liquid Chromatography (HPLC)

3.3.1 Equipment

The chromatographic Waters HPLC system, Waters 2695 Separation Module (Milford, MA, USA) was shown in Figure 3.1. It was equipped with Waters 2996 Photodiode Array Detector. The separations were performed on Waters 2695 Separation Module with HPLC Column Ascentis ® Express C18 15cm × 4.6 mm, 2.7 µm.

3.3.2 Apparatus

All the apparatus that was used for conducting this study were listed in Table 3.1.

3.3.3 Chemicals and reagent

The chemicals and reagents used in this study were of HPLC grade and listed in table 3.2.

3.3.4 Preparation of 0.03M phosphate buffer

0.03M phosphate buffer solution was prepared by weighing approximately 2.13 g of sodium phosphate dibasic then placed in Scott bottle. 500 ml of deionised water was added. The pH of the buffer solution was adjusted to pH 7.2. The acidity and basicity of the buffer solution was adjusted using orthophosphoric acid and sodium hydroxide respectively.

3.3.5 Preparation of borate Buffer pH 13

Borate buffer was prepared by mixing 2.4732 g of boric acid and 2.982 g of potassium chloride in 200 ml volumetric flask. The pH was adjusted to pH 13 using sodium hydroxide solution.

3.3.6 Mobile Phase Preparation

ACN (acetonitrile) of HLPC grade, phosphate buffer, and deionised water was freshly prepared prior to analysis. These solutions was filtered using filter system twice to make sure the solutions were free from contaminants. The filtration begin with a less polar solution followed by more polar solution i.e. acetonitrile followed by phosphate buffer and finally water. After the filtration process, the solutions was sonicated using a sonicator for approximately 10 minutes at 30ºC.

3.3.7 Preparation of Drug Standard Solutions

Ketamine standard stock solution (1 mg/ml in methanol) was diluted into 100 µg/ml using HPLC grade methanol. Internal standard i.e. morphine was also be prepared in similar manner with the ketamine standard. These drug standards was kept in amber vials and stored in a refrigerator before injecting into HPLC.

3.3.8 Injection of Drug Standard Solutions

HPLC analysis was done according to methods by Bolze and Boulieu (1998) with slight modification. The standard ketamine was first analysed at concentration of 1 µg/ml, followed by 2 µg/ml, 5 µg/ml, and 10 µg/ml. The mobile phase composed of 23% acetonitrile: 77% phosphate buffer at pH 7.2 and the flow rate was 1.0 µg/ml. For injection of morphine (internal standard), it was also analysed at the lowest concentration of 1 µg/ml followed by 2 µg/ml. Then, three flow rates of 1.0 ml/min, 0.8 ml/min, and 0.6 ml/min were tested for both ketamine and morphine (internal standard) in which the other chromatographic conditions remains constant.

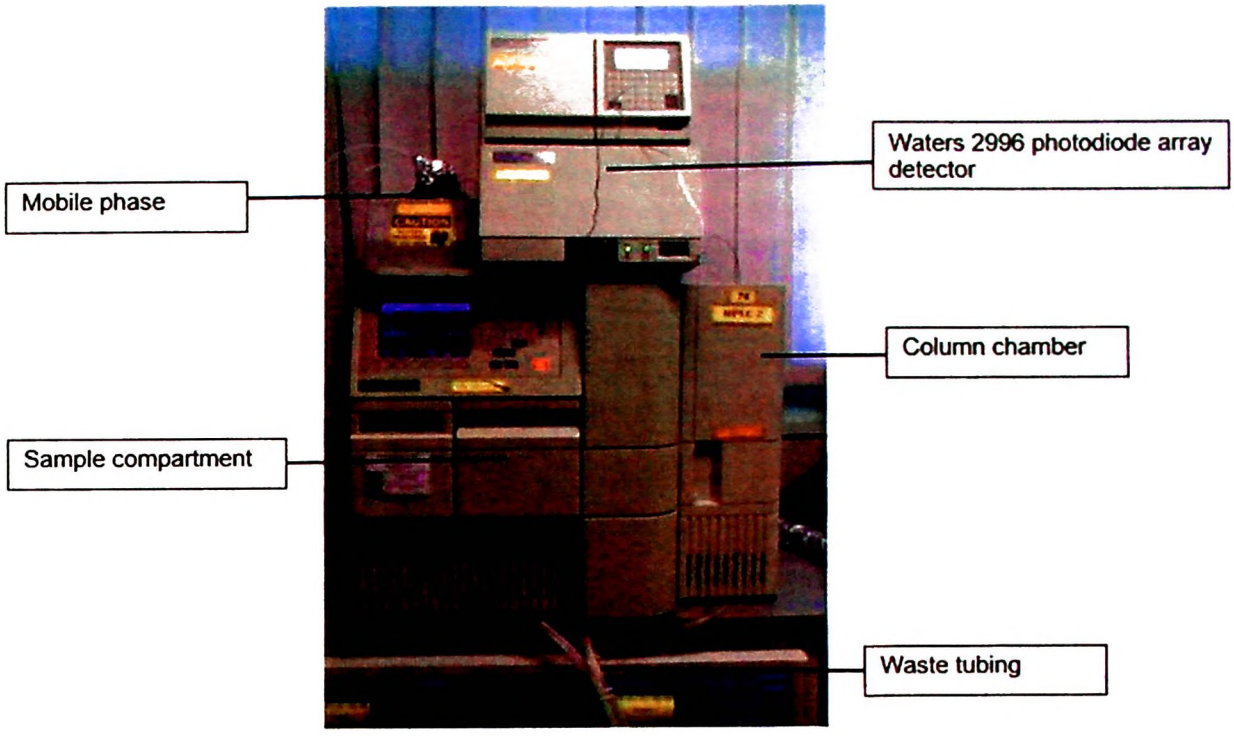


Figure 3.1: HPLC Waters 2695 Separation Module (Milford, MA, USA) used to analyse samples in this study.

Table 3.1: The list of apparatus used for HPLC analysis in this study.

Apparatus	Sources
Scott bottle	Schott Duran, Germany
Measuring cylinder	SIMAX, Spain
Pipette	Michipet EX, Japan
Beaker	Pyrex® Iwaki Glass, Japan
Analytical balance	AND, Japan
Filter system	Sartorius Stedim, Germany
Sonicator	Fisher Scientific
Vials	Chromacol, UK
Syringe	Becton Dickinson, Singapore
Vortex	Permula Sdn. Bhd., Malaysia
Glass tube	Zuelig Pharma, Malaysia
Centrifuge	KUBOTA, Tokyo, Japan
Orbital shaker	BOGGERBill, Boulevard, USA
Sartolion polyamide	Goettingen Germany
Sample dryer manifold	Pierce, Rockford
Evaporator	GE Motors&Industrial system, USA

Table 3.2 List of chemicals and reagents used in HPLC analysis

Name	Supplier
Ketamine 1 mg/ml	Cerilliant Corporation, USA
Morphine 100 µg/ml	Cerilliant Corporation, USA
Sodium phosphate dibasic	Sigma-Aldrich, Germany
Acetonitrile	Unichrom, Auckland, Australia
Methanol	Merck, Darmstadt, Germany
Dichloromethane	Fisher Scientific, New Jersey
Ethyl acetate	Sigma-Aldrich, Germany
Chloroform	Merck, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
Hydrochloric acid	Merck, Darmstadt, Germany
Nitrogen gas	M0x-linde Gases SDN BHD, Malaysia
Orthophosphoric acid	BDH, Poole, England
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Boric Acid	Ajax Chemical, Australia
Potassium Chloride	Ajax Chemical, Australia

Acetonitrile and methanol are of HPLC grade. All other chemicals and reagents used are of analytical grade.