CHAPTER ONE

INTRODUCTION

1. Opioids and Pethidine

1.1 Opioids

Opioids are drugs that have been used for thousands of years to treat pain.An opioid is a psychoactive chemical that binds to the opioid receptors which are found in specific pain-related areas such as the midbrain region, thalamus, spinal cord and primary sensitive neurons in addition to the gastrointestinal tract (Shäfer M,2010).Three different opioid receptors are known, μ, δ and κ; however, the most clinically relevant is the μ receptor.

Three different classifications of opioids, traditional, according to origin and functionional classification.

The traditional classification, based on the analgesic effect, distinguishes strong, intermediate and weak opioids. Strong opioids refer to the opioids which are pure agonists, whereas the intermediate opioids are partial agonists.

The classification according to the origin differentiates 4 main types of opioids.

Endogenous opioids are opioid peptides and are produced naturally in the body. They are also known as endorphins, enkephalins, dynorphins, and endomorphins (McCubbin JA,1993). They function in modulating different physiological mechanisms such as stress, analgesia, alcohol consumption, gastrointestinal, renal and hepatic functions, learning, memory, cardiovascular responses and other important behaviors (Vaccarino AL. *et al*.,1998)

Natural opium alkaloids are referred to as "opiates". They are naturally occurring in the opium plant, Papaver Somniferum other species and exert their effects through the binding to the opioid receptors (Németh-Zámbori É,et al.,2011).

Natural alkaloids belonging to the *Phenanthrene* class and include morphine, codeine, thebaine and narcotine while the alkaloids belonging to the *benzylisoquinoline* class

include papaverine and noscapine which have a different mechanism of action (Németh-Zámbori É., *et al*., 2011) (Brunton LL, *et al*.,2006). Morphine accounts for the highest concentration, among all other opiates, in various varieties of poppy capsule (Stranskam I, *et al*.,2013)

Esters of morphine are semi-synthetic products derived from morphine or other opiates and are considered as pro-drugs of morphine. The acetylation of morphine yields to diacetylmorphine also known as Heroin (Odell LR, *et al*., 2006).

Synthetic opioids are structurally distinct chemical products obtained in order to bind to the opioid receptors, and are also sub-classified according to the chemical structure into phenylpyperidines (pethidine, fentanyl), diphenylpropalamines (methadone), morphinans (levorphanol) and benzomorphans (pentazocine).

The classification according to the functiondescribes the pharmacological action of opioids. This includes the agonists (such as morphine), partial agonists (such as buprenorphine), agonist-antagonists (such as pentazocine) and antagonists (such as naloxone).

1.2. Pethidine

Pethidine is a strong synthetic opioid which belongs to phenylpiperidine group and is an agonist opioid (Shaumann O, 1940).

It is a potent narcotic and is used as a postoperative analgesic, a premedication for anesthesia or an analgesic during labor. It can be administered orally or parenterally, via the intramuscular, intravenous or epidural routes (Keskin HL, Keskin EA, Avsar AF, Tabuk M, Caglar GS, 2003).

Pethidine exerts its analgesic effect through interaction with μ receptor. It also binds to the κ receptor, but this binding if on no clinical significance. Due to structural similarity to atropine, Pethidine also demonstrates anticholinergic side-effects. The

stimulant effect of Pethidine is related to inhibition of dopaminergic and norepinephrine transporters. In addition, and since Pethidine interacts with serotonergic neurons, it is believed that it is associated withthe serotonin syndrome. Pethidine has a rapid onset of action and has a great potential to cause physical dependence and can lead to addiction.

1.2.1. Physical and chemical properties of Pethidine

1.2.1.1. Nomenclature

Pethidine is 1-methyl-4-phenylpiperidine-4-carboxylic acid ethyl ester commonly referred to as Meperidine Hydrochloride or Demerol® in the US*(*The Lancet, 1942).

1.2.1.2. Structure, formula and molecular mass

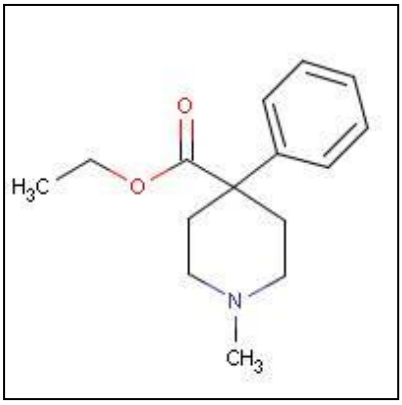


Figure 1: Pethidine structure

Formula: $C_{15}H_{21}O_2N_1$, Mwt= 247.3 (Lu *et al*., 2011)

1.2.1.3. Solubility

The fine white crystalline powder is slightly soluble in water, but the hydrochloride salt readily dissolves to form a neutral solution (The Lancet, 1942).The molecule of pethidine is basic in nature and has high lipophilic properties. The heightened lipophilicity leads to greater blood brain barrier penetration when compared to morphine(Stanski DR, 1987).

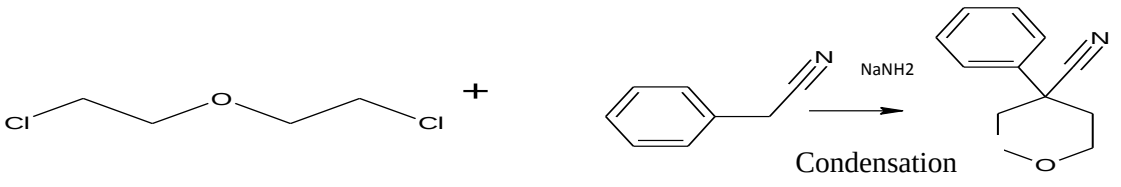
1.2.1.4. pka

Opioids are considered weak bases with their pKa ranging from 6.5 to 8.7. (Trivedi M,Shaikh S, Gwinnut C, 2007). Pethidine has a pKa of 8.5 and this is due to the free, lone pair of electrons present on the Nitrogen molecule, and Oxygen molecule (Chan K, Murray GR, Ong GC, 1980). Whereas its log p is equal to 2.72 (Gjelstad A, *et al*., 2006).

1.2.1.5. Synthesis of Pethidine

Pethidine has been synthesized through different pathways, Pethidine was synthesized for the first time by Eisnleb (Eisleb 0, 1941) through the following steps:

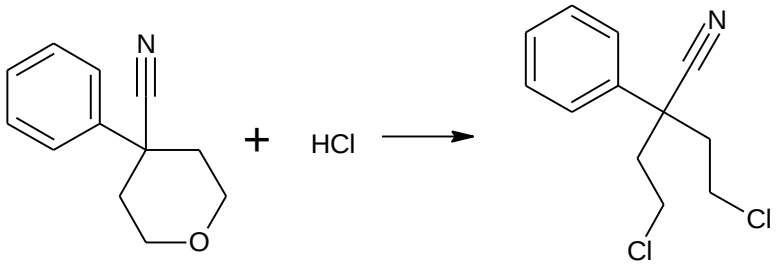
a- Condensation of β, β'-dichloroethyl ether with benzyl cyanide using sodium amide as a condensing agent. This reaction results in a Pyran derivative.



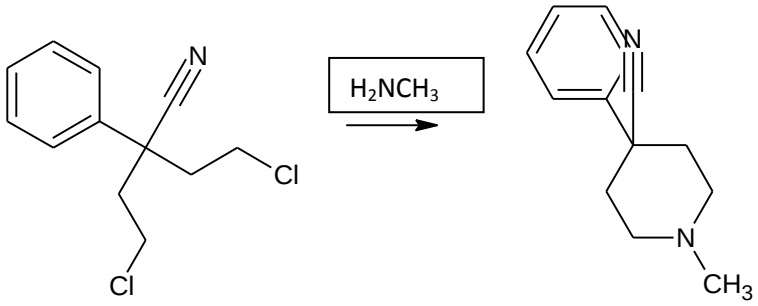
β,β'- dichloroethyl ether

Benzyl cyanide Pyran

b- Treating the Pyran derivative with halogen acid and this results in breaking the ring

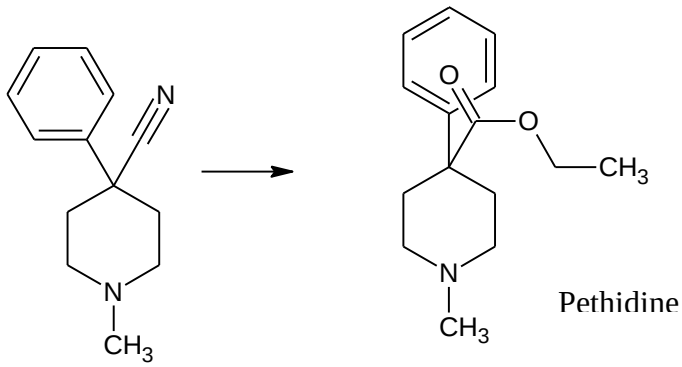


c- Treating with Methylamine in order to form piperidine ring



1-methyl-4-phenylpiperidine-4-carbonitrile

d- Replacing the carbonitrile group with ethylester group by treating with sulfuric acid and ethanol. This results in the formation of Pethidine



Pethidine

1.2.2. Method development:

The typical method of bioanalytical assay includes sample preparation, determination of the sample preparation procedure, determination of the chromatographic conditions and mass spectrometry.

1.2.2.1. Sample preparation

In order to analyze a compound in a biological matrix (e.g. blood), the sample should be treated before analysis (Mcdowall R.D, 1989). The objectives of sample preparation include:

1. Removal of the majority ofunwanted proteins that would interfere in the determination of the analyte.
2. Removal of any interference with the analyte during analysis.
3. Removal of material that would affect chromatographic resolution or reproducibility (Nakagawa T. *et al*., 1987)
4. Solubility of compound in a suitable medium in order to inject it under the initial chromatographic conditions.
5. Concentration of the analyte within the detection limits of the analytical instrument (Hagestam H., Pinkerton TC., 1985)
6. Dilution to reduce solvent incompatibility or to decrease the analyte concentration to meet the higher limit of quantification.
7. Removal of material that could block the chromatograph tubing, valve and column.
8. Stabilization of the analyte to avoid hydrolytic or enzymatic degradation.

The most widely employed biological sample preparation methodologies of Pethidine are protein precipitation (PPT), solid-phase extraction (SPE), and Liquid-liquid extraction (LLE).

The solid-phase extraction is a time and money consuming method.

The precipitation method will give rise to dilution of the solution, and consequently, lower peak intensity.

Liquid-liquid extraction will be discussed in further detail in the following section.

1.2.2.2. Liquid-liquid extraction

Liquid-liquid extraction, also known as solvent extraction, is a method of separation of the wanted analyte from a matrix on the basis of the relative solubility of the analyte in 2 immiscible liquids.

The liquid extraction of Pethidine from plasma depends on the choice of the buffer and solvent. The buffer plays a major role in increasing the extractability of the drug. Although it disappears during centrifugation, the buffer also decreases the emulsion phase that is formed between the 2 liquid phases during vortex.

As it has an effect on the physical properties of the drug (increasing its solubility in the organic solvent), the choice of the buffer depends on the acidity and basicity of the drug. If the drug is acidic, the buffer will have a low pka; whereas if the drug is basic, the buffer will have a high pka.

After adding the buffer to the plasma-containing drug, the mixture should be mixed by vortex for a few seconds.

The organic phase is then added, and the mixture will be vortexed in order to achieve the best recovery.

1.2.2.3. Internal standard:

The use of an internal standard is preferred by many analysts for quantitative analysis. The purpose of an internal standard is to minimize system and procedure variations, thus eliminating variations in precision as a function of sample size.For the proper use of an internal standard, several requirements should be met:

1. It should be completely resolved.

2. It should not elute on or over another component.

3. It should have similar chemical properties to eliminate or reduce differences in detector response between itself and the component of interest.

4.It should be of good purity to prevent adding contamination and spurious peak to the chromatogram.

5. It should be chemically inert and stable.

The addition of an internal standard makes the analysis less influenced by errors in transferred or injected volumes.

1.2.3. Validation

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduction of validation studies. The fundamental parameters for this validation include accuracy, precision, sensitivity, recovery and stability (Buick AR., *et al*., 1990.)

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the methods are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method.

1.2.3.1. Reference standard:

Analysis of drugs and their metabolites in a biological matrix is carried out using samples spiked with calibration (reference) standards and using quality control (QC) samples which are used to monitor the performance of the method and to assess the validity of the results of the unknown samples analyzed (Guidance of industry, FDA, 2001)

1.2.3.2. Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of three determinations per concentration. A minimum of three concentrations in the range of expected concentrations are recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy (Buick AR.,1990).

1.2.3.3. Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.

Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations are recommended. The precision determined at each concentration

level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV (Buick AR., 1990)

1.2.3.4. Recovery

The recovery of an analyte in an assay is the ratio between response obtained from an amount of the analyte added to and then extracted from the biological matrix, to the response obtained for the true concentration obtained by adding the same amount of analyte after extraction. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery experiments should be performed on the QCs concentrations by comparing the measured concentrations for extracted samples at three concentrations (low, medium, and high) with un-extracted standards assuring 100% recovery (Bressole F., *et al*., 1996)

1.2.3.5. Calibration standard curve:

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. The instrument response is expressed by the ratio between the AUC of the internal standard and the AUC of the analyte. A calibration curve should be generated for each analyte in the sample. A sufficient number of standards (usually 7 concentrations) should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the sample in the intended study by spiking the matrix with known concentrations of the analyte (Bressole F., *et al*., 1996).

A calibration curve should consist of a blank sample (matrix sample processed without an internal standard), a zero sample (matrix sample processed with internal standard) and six to eight non-zero samples covering the expected range, including LLOQ.

The calibration curve linearity is expressed by the R^2 which should have a value exceeding 0.996.

1.2.3.6. Stability:

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system used during the method of analysis (Ventura R. *et al*., 2003)

Stability procedures should evaluate the stability of the analyte on three levels, bench top storage, autosampler storage, and after going through three freeze and thaw cycles during the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis.

1.2.3.6.1. Freeze and thaw stability:

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low, medium and high concentrations of QC samples should be stored at the intended storage temperature for 24 hours and thawed at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions (Ventura R. *et al*., 2003).

The freeze-thaw cycle should be repeated two more times, and then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the samples should be frozen at –30°C during the three freeze and thaw cycles.

1.2.3.6.2. Bench top stability:

Three aliquots of each of the low, medium and high concentrations of QC samples should be thawed at room temperature and kept at this temperature from 0.0 to 6.0

hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and then analyzed (Ventura R. *et al*., 2003).

1.2.3.6.3. Autosampler stability:

The time spent on the autosampler by the analyte should exceed the time between the moment of first sample injection and the last sample injection when multi injections are used during analysis. Autosampler stability should be determined by storing at least three aliquots of each of the low, medium and high concentrations of QC samples under the same conditions on the autosampler for 24 hours.

The volume of samples should be sufficient for analysis on two separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of autosampler stability testing (Ventura R. *et al*., 2003).

1.2.3.7. Sensitivity

The sensitivity defines the lowest amount of the analyte that the method used can detect. During validation, the sensitivity determines the efficiency of the method for the detection of the lower limit of detection used in the calibration curve.

The sensitivity is expressed by the accuracy and precision of the results after injection of three of each of the low, mid and high concentrations.

1.2.4. Determination of Pethidine in human plasma in the previous studies

The efficiency of the method of analysis of Pethidine in plasma is related to the following:

- Efficient extraction from plasma
- Efficient separation from other components
- Less interference and high selectivity

The methods of analysis used for the determination of Pethidine:

1.2.4.1. Liquid chromatography followed by tandem mass spectrometry

Tandem mass spectrometry is defined as coupling of a series of mass spectrometers. This method has proven to possess very high selectivity and sensitivity (McLafferty FW, 1981). The first mass spectrometer is responsible for ionizing the analyzed molecule resulting in the primary ions which are separated from the mixture. The secondary ions of the compound, which are the products of the primary ions collisions, are then selectively determined by the second sass spectrometry.

The disadvantage of this method is time consuming, around 6 minutes each run and the validated Pethidine concentration ranges from 4 to 2000 ng/ml (Wang X *et al*., 2011).

In the tandem mass spectrometry, triple quadrupole can also be used in order to increase selectivity and sensitivity of the analysis (Smyth WF, Joyce C, Ramachandran VN, O'Kane E, 2004). The ionization is performed in the region of the source where the ions are separated according to m/z ratio then undergo collision induced decomposition and the mass is analyzed (Jiao J, Carella AJ, Steeno GS, Darrington RT, 2002).

1.2.4.2. Ultra performance liquid chromatography followed by tandem mass spectrometry

. The main parameters that play a major role in reaching high sensitivity in this method are the alkaline wash in the solid phase extraction, the high alkalinity of the mobile phase, the electron spray ionization and the 1.7 µm particle size of the column. The linearity of the calibration curve is achieved from the concentration of 0.5 to 40 ng/ml of whole blood (Verplaetse R, Tytgat J., 2012).

1.2.4.3. High performance liquid chromatography followed by photodiode array ultraviolet

This is used for the identification and separation of classes of drugs in plasma and is not used for quantification. In fact, the pholodiode array ultraviolet (DAD) component allows the possession of the UV spectra of the compounds (Hayashida M., Nihira M., Watahabi T, Jinno K, 1990). This method mainly relies on the retention index (RI) in order to consider the variability of the retention time for each compound over the whole period of analysis> This variability occurs as result of a gradual loss of the stationary phase (Elliot SP, Hale KA, 1998). This method utilizes a solid phase extraction (SPE) with C18 sorbent and as such, is a time and material consuming method (Alabdallah MA, 2005).

1.2.4.4. Exhaustive electro-membrane extraction followed by liquid chromatography-mass spectrometry

The objective of electro-membrane extraction (EME) is to separate the basic compound from the matrix by the means of electro-kinetic migration through

supported liquid membrane (SLM) fixed in a hollow fiber towards the cathode which is placed in the acceptor solution (Pedersen-Bjergaard S, Rasmussen KE, 2006). The exhaustive EME is based on increasing the number of hollow fibers from 1 to 3, which provides a recovery in the range of 97-115% for spiked solution and 56%-102% for human plasma. However, it should be noted that in the latter percentage range, the duration of extraction was 60 minutes (Eibak LEE, Gjelstad A, Rasmussen KE, Pedersen-Bjergaard S, 2012).

1.2.4.5. Gas chromatography followed by mass spectrometry

In this method, the extraction of Pethidine from whole blood was performed using Bond Elut C_{18}, the recoveries of Pethidine were 109% ±STD = 17.1 and ±STD= 10.9 for concentrations of 4ng/ml and 20ng/ml, respectively. The linearity ranges between concentrations of 1.25ng/ml and 40ng/ml. Disappointingly, this method showed high intra-day and day-to-day variations and the C.V. values were above 10% (Ishii A *et al*, 2003).

1.2.4.6. Gas chromatography linked to specific nitrogen detector

The nitrogen detector, also called a thermionic detector, is a very sensitive and can be used as a specific method for detection. It is based on the use of thermal energy to ionize an analyte; namely nitrogen present in Pethidine. The sensitivity of this method reaches 5ng/ml of Pethidine in plasma (Szkutnik D, Dyderski S, Majcher K, 2001),(Tse J, Chan K, 1981). Another gas chromatography method described in the literature is the capillary gas chromatography which entrails a capillary glass column of 3mmX0.32mm dimension (Szkutnik D, Dyderski S, Majcher K, 2001).

1.2.4.7. Gas chromatography with electron capture detection

This method of detection is reported to be 10-1000 times more sensitive than flame ionization detectors. It detects components with high electro-negativity. Consequently,Pethidine requires treatment prior to analysis. This is usually done with trichloroethyl chlorformate to produce trichloethyl carbamate (Hartvig P, Karlsson K.E, Lindbert C, Boréus LO, 1977).The sensitivity of this method reaches only 100ng/ml Pethidine in plasma, (Hartvig P,et al., 1976).

1.2.4.8. Fluorimetric procedure

This method has also been used for the determination of Pethidine but the limit of quantification is just 300ng/ml of blood, (Hartvig P,*et al*., 1976).

1.2.4.9. Capillary electrophoresis with electro-chemi-luminescence detection

The detection is performed using tris(2,2-bipyridyl)ruthenium(II) (Ru(bpy)32+). Pethidine is injected into a separation capillary by electro-kinetic injection and under optimized conditions.The linear range is realized from2.0×10^{-6} to 2.0×10^{-5} M and the detection limit is achieved at 0.5μM (Han B, Du Y, Wang E,2008).

2. Analgesia in Labor

Maternal stress resulting from labor induces the release of massive amounts of maternal cortisol and catecholamines in addition to the release of catecholamines in the fetus (Reynolds F, 2010).

The release of catecholamines in the fetus enhances the blood circulation in the heart, adrenal and the brain, aids in the development of adaptation to the circulatory changes after birth and stimulates the release of surfactant.

On the other hand, maternal catecholamines and cortisol may lengthen the labor process and weaken the placental blood flow(Lederman RP, *et al*.,1978),(Ohno H, et al., 1986) and(Sega lS, Wang SY, 2008).

Stress hormones initiate lypolysis and liberation of free fatty acids which are transferred through the placenta and hyperglycemia as a consequent exacerbation of fetal hypoxia. All alterations, which become more severe as labor progresses, result in an amplification of the fetal metabolism(Thalme B, *et al*., 1974).

In addition, maternal hyperventilation during labor results in respiratory alkalosis and left shift of the oxygen dissociation which impairs the transfer of oxygen to the placenta. Compensatory metabolic acidosis ensures which is transmitted to the fetus(Thalme B, Belfrage P, Raabe N, 1974). Furthermore, hyperventilation is associated with uterine vasoconstriction and episodes of hypoventilation between contractions (HuchR, 1986).

Consequently, an effective and safe analgesia given during labor is beneficial to the mother and the newborn. In fact, adequate relief of pain during the first stage and in the early portions of the second stage of labor and during the perineum repair help

keep the mother cooperative during delivery, increase the for normal vaginal delivery and decrease the need for forceps or suction for extraction of the baby(Harry SF,1954). High neonatal Apgar score, with unimpaired acid-base balance, neurobehavioral status, respiratory status and breast feeding response are important in the assessment of the condition of the newborn.

Nevertheless, most compounds that crossthe blood-brain barrier readily cross the placenta; so after a given time period, the administered analgesic would eventually affect the baby(ReynoldsF, 1991).

In 1853, it was shown that high doses of chloroform produce a sleepy baby and Snow suggested to monitor the chloroform dose (SnowJ, 1953).

In the early 20th century, the use of hyoscine as sedative followed by barbiturates has been proven to be harmful to the mother as well as the baby. Barbiturates are not true analgesics, they tend to cause sleep within less than half an hour following administration and are likely to initiate restlessness and delirium (Fritsch JE, Brown R, 1935). Since they depress the central nervous system, barbiturates result in a decrease and disruption of breathing rhythm. Fetal transfer, via the placenta, could result in somnolence, flaccidity and bradycardia (Galloway CE *et al*., 1936)

In mid-20th century, nitrous oxide and Pethidine were introduced as analgesics in management of labor pain instead of old sedation regimens.

Nitrous oxide is a volatile analgesic administered through inhalation. It results in less cumulative deposition in baby as it is readily excreted via lungs once born. In order to obtain maximal analgesic effect, the tendency of the hyperventilation is promoted which leads to hypocarbia resulting in oxygen desaturation. Entonox, a mixture of

50% nitrous oxide and 50% oxygen, was then developed with the aim of preventing this problem (Tunstall ME,1961).

Pethidine, first synthesized 1939, has been used as an agent in labor analgesia since 1940 (Bricker L, Lavender T, 2002).Since then, Pethidine has become the most widely used opioid for labor analgesia. It was reported that the use of Pethidine for labor analgesia in England in 1990 was 37% and in the United States it was estimated between 39% and 56% (Sosa CG, *et al*., 2006). Studies addressing Pethidine raised reservations concerning its effectiveness in the management of labor pain and potential unwanted effects on the delivery process and on the newborn since it was clear, at the offset, that Pethidine readily crossed the placenta (Reynolds F, 2010).The analgesic plasma level of Pethidine, during labor ranges from 200ng/ml to 400ng/ml. Previous studies evaluating the effect of Pethidine on the newborn demonstrate that it results in depression of neonatal vital signs such as breathing movements, heart rate, respiratory rate and consequently Apgar score (Scrutton M,1997). Agar score used as a baseline assessment of the health and wellbeing of the neonate at 1 to 5 minutes after birth (Blake D, 2010.). Apgar score is calculated by giving a score ranging from 0 to 2 for the following factors:

- Appearance: evaluating the skin color
- Pulse: evaluating the heart rate
- Grimace: evaluating the reflex after stimulation
- Activity: evaluating the muscle tone
- Respiration: evaluating the respiratory rate

The aim of this study is to determine the concentration of Pethidine level in woman blood at different times during the labor, and the concentration of Pethidine in the neonatal cord just after delivery following a 50mg intravenous bolus injection. We

will attempt to highlight possible relationships between different factors such as dose, time of administration, mother weight, fetal condition, newborn weight, newborn sex and on the Apgar score.

CHAPTER TWO

METHODOLOGY

1. Analytical Part

1.1.Chemical reagents and instrumentations

- Pethidine (Manufacturer: Martindale, batch No: 00482, production date 09/2010 and expiry date 09/2013); Methanol (Fisher Lot:1155904);H_2O (Fisher Lot: 1207702); Ethyl acetate (Fisher Lot: 1080888) ; Diethyl ether (Tedia Lot: 509151); TCA (Acros Lot: A0316403, 99%); Formic Acid (VWR Lot: 07L210512); Ammonia (Fisher Lot: 0804270, 93%); Di-chloro-methane (Acros Lot:0878339); Methyl tertiary butyl ether (Fisher Lot: 1293152); NaOH (VWR Lot: B0057050); Na_2CO_3 (Merck Lot:A939493 904); Propranolol HCl (APM ws HS12019 Lot 10212455); Bisoprolol Fumarate (RD ws 304, 99.7%).
- Balance (Sartorious BP2215); Centrifuge (Eppendorf, 5702R); pH meter (Sartorious, PP-25).
- Ion trap Mass spectrometer; Constant solvent delivery system (Ultimate 3000 RS Pump); 130µl fixed volume injector; Online vacuum degaser (Ultimate 300RS); Autosampler (Ultimate 3000 RS autosampler); Column: ACE 5 C_{18} 50x2.1mm; LCQ Fleet Mass Spectrometer; Computer system (Windows XP, Thermoscientific®, X Calibar®, Data management software).

1.2. Method development

1.2.1. Tuning of Mass Spectrometer

The tuning was performed with a 5 µg/ml solution of Pethidine. The solution was prepared using the 50mg/ml standard solution.

A standard solution A of 50 µg/ml was first prepared by diluting 10µl from the standard solution of 50 mg/ml in q.s.10ml 1:1 H_2O/MeOH (Methanol). Then 1ml of the standard solution A was diluted in q.s.10 ml 1:1 H_2O/MeOH to obtain a 5µg/ml solution of Pethidine.

200 µl were transferred into insert vials and injected into LC-MS using the following parameters:

Mobile phase: Tri-Chloro-Acetic Acid +Ammonia at pH=3 as aqueous phase 40%

Methanol 60%

Flow rate: 0.20 ml/min

Injection Volume: 5µl

Temperature of auto sampler: 25°C

1.2.2. LC conditioning

The effect of chromatographic conditions on the separation of pethidine, pH, composition of mobile phase and column was studied to select a proper method for quantification of Pethidine in biological fluid to improve the stability.

Chromatographic conditions were developed as indicated in the table 2.1.

A solution of 1000ng/ml was prepared by diluting 200 µl of the standard solution A (50 µg/ml) in 10 ml q.s.10ml 1:1 H_2O/MeOH.

Table 2.1: Chromatographic conditions

		Method 1	Method 2	Method 3
Mobile phase	Aqueous phase	Formic acid 30%	TCA 30%	TCA 30% + Ammonia
	Organic phase	Methanol 70%	Methanol 70%	Methanol 70%
pH		2.5	3	3
Auto-sampler Temperature		25°C	25°C	25°C
Flow rate		0.2 ml/min	0.2 ml/min	0.2 ml/min
Injection Volume		5 µL	5 µL	5 µL

1.2.2.1. Effect of the aqueous phase and pH

Three different aqueous phases were used in order to choose the most appropriate resolution and intensity of Pethidine peak. Since, Pethidine is a basic drug, the pH of the mobile phase is chosen to be low in order to increase its solubility. The higher portion of organic phase in the mobile phase also helps in increasing the solubility of Pethidine.

1.2.3. Extraction method

Liquid-liquid extraction method was studied by evaluating the effects of the solvent and buffer.

The following solvents were studied:

- Ethyl acetate
- Diethyl ether
- Methyl tertiary butyl ether

- Dichloro-methane + ethyl ether (30:70). It should be noted that dichloro-methane has higher intensity than plasma, therefore ether is added to decrease the density and the organic layer would be supernatant.

The following buffers were evaluated:

- NaOH: 1 M
- Na_2CO_3: 1 M

The liquid-liquid extraction methods were studied as described below:

12 different solutions of 250 µl plasma-spiked with Pethidine were used in order to obtain 1000 ng/ml plasma solution.

4 solutions using NaOH as buffer with each solvent added on every solution.

4 other solutions using Na_2CO_3. In the final 4 solutions, no buffer was used.

In each Pethidine plasma solution, 1ml of the buffer was added and then vortexed for 5 -10 seconds in order to achieve a homogenous solution. Even in the solutions where no buffer was added, these were vortexed to decrease the error probability among the 12 solutions.

6ml of the solvent were added to the solution and vortexed for 5 minutes, since this time determines the amount of Pethidine extracted in the organic phase.

The solutions were centrifuged for 6 minutes on a speed of 4400 rpm.

Decantation was performed using a Pasteur pipette and the organic phase was transferred into glass tubes. The solutions were evaporated at 40°C in water bath under nitrogen gas for 30 minutes.

The residue obtained was reconstituted in 200 µl H_2O/Methanol (2:1).

The choice of the reconstitution medium depends on:

- The stability of the drug in the medium chosen
- The solubility of the drug in the medium
- The technique which was chosen for detection.

After reconstitution, the solution was vortexed for 1 minute to ensure complete dissolution and it was transferred into insert tubes, and then centrifuged to eliminate the turbidity waste. The solution was transferred into vials and then injected in the LC-MS/MS.

1.2.4. Selection and preparation of the internal standard

1.2.4.1. Propranolol

Propranolol is a basic and non-polar drug; the chemical structure and nomenclature are shown in the figure 2.1.

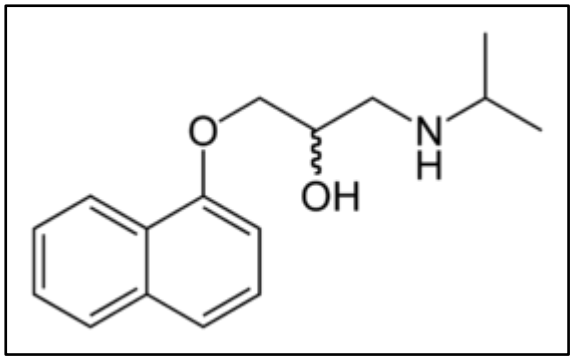


Figure 2.1: Propranolol;1-[(2-methylethyl) amino] - 3 - (1-naphthalenyloxy) - 2 – propanol (Bartolomei M, Bertocchi P, Ramusino MC, Santucci N, Valvo L, 1999)

A stock solution of propranolol was prepared by dissolving 10 mg in 10 ml solution of 1:1 H_2O/Methanol producing a 1000 µg/ml propranolol solution.

0.5 ml from stock solution was withdrawn using a pipette and diluted in 1:1H_2O/Methanol q.s. of 100 ml to obtain 5 µg/ml propranolol solution.

200µl was transferred into a vial and injected in the LC-MS/MS system using C.E =38.

1.2.4.2. Bisoprolol

Bisoprolol is a drug of the same class of Propranolol but with higher polarity; the structure and nomenclature are shown in figure 2.2.

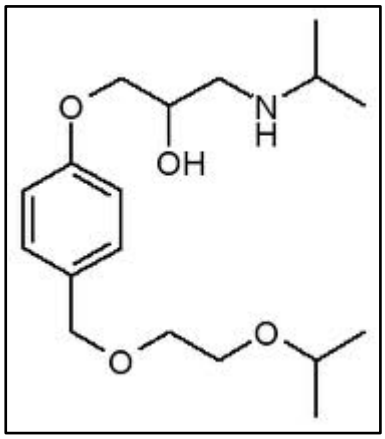


Figure2.2:Bisoprolol;1-{4-[(2-isopropoxyethoxy)methyl]phenoxy}-3-(isopropylamino) propan-2-ol

A stock solution of Bisoprolol was prepared by dissolving 10 mg in 10 ml solution of 1:1 H_2O/Methanol producing a 1000 µg/ml Bisoprolol solution.

0.5 ml from stock solution was withdrawn using a pipette and diluted in 1:1H_2O/Methanol q.s. of 100 ml to obtain 5 µg/ml bisoprolol solution.

200 µl was transferred into a vial and injected in the LC-MS/MS system using C.E =38.

1.2.5. Adjusting the retention time

The retention time of Pethidine and bisoprolol are slightly high; 2 factors should be adjusted:

- The composition of the mobile phase.

If the polarity of the mobile phase is decreased, the solubility of the analyte will increase relatively to its affinity to the column and eventually, the retention time will decrease. As a consequence, the concentration of methanol in the organic phase was increased to 80%.

- The flow rate

Increasing the flow rate to 0.3ml/min helps in eluting the analyte from the column in less time

The method of analysis used is described in the table 2.2

Table2.2: Conditions of the method of analysis

				Source	
Liquid-liquid extraction	buffer		no buffer		
	solvent		methyl tertiary butyl ether	Fisher lot: 1293152	
	vortex		5 mins		
	centrifugation		6 mins on 4400 rpm	eppendorf 5702R	
	reconstitution		200 µl 2:1 H2O/Methanol	H2O;Fisher Lot: 1207702 Methanol;Fisher lot: 1155904	
Internal Standard	Bisoprolol		Bisoprolol fumarate	RD-WS304 99.7% manufactured: 28/12/2012	
Liquid chromatography	Mobile phase	aqueous phase	TCA + Ammonia	TCA; 99% Acros lot:A0316403 Ammonia; 35% Fisher lot	1. Constant system delivery system (ultimate 3000RS pump) 2. 130mcl fixed volume injection 3. Online vacuum degasser (ultimate 3000 RS) 4. Autosampler (ultimate 3000 RS) 5. LCQ Fleet Mass Spectrometer 6. Computer system, windows XP, thermoscientific®, xcalibar®, data management software
		organic phase	Methanol 80%-H2O 20%	H2O;Fisher Lot: 1207702 Methanol;Fisher lot: 1155904	
	pH		3		
	Auto-sampler Temperature		25°C		
	Flow rate		0.3ml/min		
	Injection Volume		5µL		
Mass spectrometer	tandem mass specrometer		Ion trap detector		

1.2.6. Preparation of the standard curve and QCs

A stock solution of Pethidine was prepared by diluting 1ml of the standard 50mg/ml to q.s. 100 ml 1:1 H_2O/Methanol, resulting in 500 µl/ml solution. The latter was used to prepare 7 of 10 ml working serial solutions and 3 of QC solutions of the following concentrations:

The serial standard concentrations: 20 ng/ml, 40 ng/ml, 80 ng/ml, 150 ng/ml, 300 ng/ml, 600 ng/ml and 1000 ng/ml.

The QC concentrations: 60 ng/ml, 500 ng/ml and 800 ng/ml.

The above solutions were used to prepare the spiked plasma as described in the tables 2.3 and 2.4.

Table 2.3: Preparation of spiked plasma for calibration curve

Solution No:	Working Solution Concentration (µg/ml)	Stock conc. (µg/ml)	Volume taken from stock (µl)	Total Volume (mL)	Cal ID	Volume taken from w.s (µl)	Total Volume (ml)	Final Concentration (ng/ml)
S1	**1.0**	**500**	**20**	**10**	**C1**	**200**	**10**	**20**
S2	**2.0**	**500**	**40**	**10**	**C2**	**200**	**10**	**40**
S3	**4.0**	**500**	**80**	**10**	**C3**	**200**	**10**	**80**
S4	**7.5**	**500**	**150**	**10**	**C4**	**200**	**10**	**150**
S5	**15.0**	**500**	**300**	**10**	**C5**	**200**	**10**	**300**
S6	**30.0**	**500**	**600**	**10**	**C6**	**200**	**10**	**600**
S7	**50.0**	**500**	**1000**	**10**	**C7**	**200**	**10**	**1000**

Table 2.4: Preparation of QC spiked plasma

Solution no:	Working Solution Concentration (µg/ml)	Stock conc. (µg/ml)	Volume taken from stock (µl)	Total Volume (mL)	QC ID	Volume taken from w.s (µl)	Total Volume (ml)	Final Concentration (ng/ml)
S11	**3.0**	**500**	**60**	**10**	**QC LOW**	**500**	**25**	**60**
S12	**25.0**	**500**	**500**	**10**	**QC mid**	**500**	**25**	**500**
S13	**40.0**	**500**	**800**	**10**	**QC high**	**500**	**25**	**800**

Each concentration of the spiked plasma was divided into 250 µl aliquots in test tubes and were kept in the freezer at -30°C.

1.3. Validation

Validation was performed in order to evaluate the method in terms of sensitivity, stability, recovery, accuracy, precision and linearity of response. The validation was performed in three separate days with a seven point standard line (not including zero) and three points of (low, medium, high) QC samples in each day. Each day of validation included plasma samples representing three complete standards and QC sample lines.

1.3.1. Sensitivity

The sensitivity reflects the efficiency of the method engaged to detect the lower limit of quantification (20ng/ml pethidine in plasma).

Ten solutions of the lower limit concentration (20ng/ml spiked plasma)were injected into the LC-MS/MS. The measured concentration was calculated, as well as the

accuracy and precision. The precision and the accuracy values should be within the range ±20%.

1.3.2. Stability

The stability study evaluates the stability of the analyte after the sample preparation in the autosampler, on the bench and after 3 freeze and thaw cycles. This validation was performed only on the QC samples.

1.3.2.1. Autosampler stability

The autosampler stability study evaluates the stability of Pethidine during the injection in the autosampler held at 15°C. The validation is performed over a duration of 24 hours. 50 µl of the internal standard stock solution was added to three of each 250 µl melted QC aliquot, the mixture wass vortexed. After the extraction procedure and reconstitution with 200 µl 2:1 H_2O/Methanol, the solutions were transferred into vials and placed in the autosampler. They were injected and kept for 24 hours in the autosampler and then re-injected into LC-MS/MS.

1.3.2.2. Freeze and thaw stability

This validation study is based on the evaluation of the stability of the analyte after 3 cycles of melting and freezing the Pethidine sample. The cycle consists of melting the freezed Pethidine sample for 30 minutes and then re-freezing it for 24 hours without the presence of an internal standard. This study was performed on the QC samples. After 3 cycles of freeze and thaw (72 hours), 50 µl of the internal standard was added

and the mixture was vortexed. After extraction and reconstitution according to the accredited method, the samples were transferred into vials and injectedinto LC-MS/MS.

Three samples of each QC concentration were prepared, extracted and reconstituted and then injected into the LC/MS-MS.

The samples which had been freezed and thawed and the samples which were injected at zero time were compared. The accuracy should have a range limit of 15%.

1.3.2.3. Bench stability

The bench stability validation evaluates the stability of the analyte after being kept on the bench at room temperature for 6 hours. It was performed on the QC samples only.

Six samples of each QC concentration were prepared according to the described method. Three of each were injected at zero time and the others were kept on the bench at 25°C for 6 hours and then re-injectedinto the LC/MS-MS..

The 2 groups of each concentration were compared, and the accuracy deviation should not exceed 15%.

1.3.3. Recovery

The extraction efficiency was determined by measuring the absolute peak area of Pethidine and the internal standard from a QC plasma samples which were prepared according to the method of analysis. QC samples containing different concentrations (60, 500 and 800ng/ml) were prepared in plasma and contain 50 µl taken from 5 µg/ml

internal standard solution assuring 100% recovery, these samples were defined as spiked plasma.

The absolute peak areas obtained from the injections of the prepared QC plasma samples were compared to the absolute peak areas of an equivalent spiked plasma QC's. The recoveries of Pethidine and Bisoprolol were calculated by dividing the mean ratio of the QC in plasma to the mean ratio of the QC of spiked plasma.

1.3.4. Matrix effect

The effect of the matrix on the analysis of Pethidine was determined by measuring the absolute peak area of Pethidine and the internal standard from a spiked QC plasma samples which were prepared according to the method of analysis. QC samples containing concentrations of (60, 500 and 800) ng/ml, were prepared in 2:1 H_2O/Methanol and contain 50 µl which was taken from 5 µg/ml internal standard solution assuring no effect of the matrix.

The absolute peak areas obtained from the injections of the prepared QC plasma samples were compared to the absolute peak areas of equivalent solutions of Pethidine and internal standard in 2:1 H_2O/Methanol. The matrix effect on Pethidine and Bisoprolol was calculated by dividing the mean ratio of the QC in plasma to the mean ratio of the QC of the solutions.

1.3.5. Linearity

The linearity of the method was tested by weighted least squares regression analysis of standard plots associated with 7points. Plotting of peak area ratios of standards versus concentration for each sample set and the calibration standard lines were shown to be linear from 20 to 1000 ng/ml for Pethidine plasma. R^2 should be less than 0.0996.

1.3.6. Precision

The precision of the assay was measured by intra-day and inter-day percent coefficient of variation over the concentration range from 20 to 1000 ng/ml for Pethidine during the course of the validation. Precision was estimated in 10 samples for each quality control concentration and was repeated for 2 consecutive days.

1.4. Sample preparation

The blood samples taken from each patient were withdrawn in heparinized tubes and kept in an ice box till centrifugation. A maximum of 6 hours was allowed between sampling and centrifugation.

After centrifugation, the plasma was withdrawn with Pasteur pipette in an Eppendorf tube and kept in the freezer at - 30°C till the validation of the method.

On the day of the sample analysis, a calibration curve was performed with 7 standard points, from which the calibration equation was deducted.

The patient samples were melted and prepared according to the described method and injected into the LC/MS-MS. The concentrations were back calculated from the equation.

2. Clinical Part

2.1. Design

The study was conducted according to an open, single dose, one period, one treatment in pregnant women in labor. A total number of 12 pregnant women inlabor had completed the trial.

Choice of pregnant women: The objective of this study was based on selection of pregnant woman who were already intended to take this analgesic medicatiom.

Duration of the study: The study would continue for a period of 1 month starting from the date of agreement given by Ministry of Health.

Choice of dosage: The dosage of the test product (50 mg/ml IV pethidine HCL) which is the recommended dose given to women during labor at Al-Bashir Hosptial.

2.2. Selection of patients

12 pregnant women, who fulfilled the inclusion criteria, did not harbor any of the exclusion criteria and who provided written informed consent, were enrolled in this study.

Drug assays and statistical analysis of the data was performed on all plasma samples of patients irrespective of completionof the protocol.

2.2.1. Inclusion criteria

Pregnant women presenting with all of the following criteria were enrolled in this study:

- Age between 16 and 45 years.
- Physically and mentally healthy as judged by means of a medical and standard laboratory examination.
- No previous alcohol consumption or history of alcoholism or drug/chemical abuse.
- Informed consent provided in written form.

2.2.2. Exclusion criteria

Pregnant women presenting any of the following were not included in this trial:

- Allergic diathesis or any clinically significant allergic disease
- History of allergic response to Pethidine.
- Presence or history of liver disease, periphieral edema, cardiovascular disease or cardiovascular risk factors.
- Severe renal insufficiency.
- Participation in another clinical trial within the last 2 months.
- Donation of blood or plasma within the last 2 months.
- Clinically relevant abnormalities at physical examination or laboratory tests.
- Any chronic disease which might interfere with absorption, distribution, metabolism or excretion of the drug.
- Intake of phenytoin, rifampicin, phenobarbitone or mono-amino oxidase inhibitors within 2 weeks prior to the start of the study.

- Intake any other anesthetic or narcoticduring delivery.
- HIV infection or carrier of the respective antigens
- Evidence of an uncooperative attitude.
- Legal incapacity and/or other circumstances rendering the patient unable to understand the nature, scope and possible consequences of this study.

2.3. Safety Assessment

All pregnant women involved in this study were included in the listing of the safety data. Reason and date for any withdrawal were reported. All adverse events were listed and tabulated by severity, treatment and relationship to the intervention.

2.4. Study Procedure

2.4.1. General Procedure

Pregnant women within the age limits were questioned with reference to the inclusion and exclusion criteria. Patients suitable for inclusion were requested to provide an informed consent and their code was recorded in the Screening List.

The patient's laboratory tests were recorded if available before the trial during her last visit to the doctor.

A list of the normal ranges and units of measurement of the laboratory parameters were determined during the study and documented in each CRF. Any value out of range was assessed as "not clinically relevant" (NCR) or "clinically relevant" (CR) on the laboratory printout.

If during the course of entry screening, any clinical relevant abnormal value had been observed, this finding was regarded as an exclusion criterion. Single laboratory values outside the normal range will generally not be regarded as an exclusion criterion provided that:

1. They are not accompanied by clinical symptoms,

2. The context of related laboratory values gives no indication of a pathological process and,

3. The investigator regards them as "not clinically relevant" (NCR).

2.4.2. Daily activities in each period of the trial

2.4.2.1. Entry examination

The entry examination is carried at the time of patient admission to the delivery room.

The following parameters are documented and /or investigated:

- Thorough explanation of the study medication from the investigators
- Date of inclusion (date of signing the informed consent),
- Date of examination,
- Inclusion criteria (according to protocol),
- Exclusion criteria (according to protocol).
- Informed consent (if inclusion criteria fulfilled).
- Demographic data: date of birth, ethnic group, sex, height, weight and BMI.

- Additional information concerning illness within the last 4 weeks prior start of the trial, lastparticipation in any clinical trial, last donation of blood or plasma, last administration of any medication (including OTC or topical medication), last administration of drugs known to alter the major organs or systems as well as specification of the drug name, dosage, start and end of treatment.

- Vital signs: registration of body temperature, measurements of supine heart rate (HR) and blood pressure and respiratory rate after 5 minutes of rest,

- Physical examination

2.4.2.2. Blood Sampling

The following procedures were performed:

- A venous canula was inserted and 2.00 ml blood sample is drawn pre-drug administration.
- Blood sampling was perofomed at the following times: immediately after labor from mother and 2 hours post drug administration.
- A blood sample of 2.00 ml was withdrawn from the umbilical cord after child birth.

2.4.2.3. Drug Administration

The precise instructions for drug administration were given to patients by the investigators.

All patients, except patient 1, received a single dose of 50 mg/ml of IV Pethidine. Patient 1 had received 2 different doses of Pethidine in 2 hours interval.

2.4.2.4. Blood Sample Storage for Drug Analysis

2ml blood samples were collected into tubes using heparin as an anti-coagulating agent. After sampling, the tubes were placed in an ice box till the end of each day and then centrifuged (at a speed of 4000 rpm at room temperature for 5.0 min). The separated plasma was transferred into polypropylene tubes which were immediately frozen and stored at a temperature of -30^0 C until analysis.

2.5. Analysis

The determination of Pethidine plasma concentrations were performed by means of LC-MS/MS chromatographic assay method at the laboratories of Jordan Centre for Pharmaceutical Research (JCPR), Amman, Jordan. A validated method for Pethidine analysis was worked out before the analysis of the patients' samples.

The method was been validated for linearity, sensitivity, stability, recovery, precision and accuracy.

2.6. Biometrics and statistical aspects

Biostatistical evaluations were carried out using data management unit by means of the software; Kinetica 2000 (version 4.1, Innaphase Corporation, France).

2.7. Data Presentation

The concentration vs. time profiles after administration of IV Pethidine were represented in the form of tables and graphics for each pregnant women and the concentration of Pethidine in the newborn infants were also measured and tabulated.

2.8. Ethical Considerations

2.8.1. Declaration of Helsinki

The study has been performed in accordance with the relevant articles of the Declaration of Helsinki (1964) as revised in Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West, RSA (1996) and Edinburgh (2000), the "Note of Clarification on Paragraph 29" added by the WMA General Assembly, Washington (2002), the "Note of Clarification on Paragraph 30" added by the WMA General Assembly, Tokyo (2004) (Note of Clarification on Paragraph 30 added), and the 59th WMA general Assembly, Seoul, October 2008.

2.8.2. Ethical, Legal and Administrative Aspects

Prior to the initiation of the study, the protocol, the patient information leaflet and the informed consent form were submitted to the Ethics Committee (IRB/IEC) responsible for the review and approval at the Ministry of Health and Al-Basheer Hospital where the study was carried out.

The study was only performed when full approval was obtained from the IRB/IEC and a copy of the certification was received, including a list of the actual members of the IRB/IEC.

CHAPTER 3

RESULTS

AND DISCUSSION

1. Method Development and Validation

1.1. Method development

1.1.1. Tuning of spectrometry

After the injection of Pethidine solution, 2 peaks appeared: 248.2 m/z parent and 220.1 m/z daughter.

1.1.2. LC conditioning

1.1.2.1. Effect of aqueous phase and pH

The 3 methods described in table 2.1 shows three different aqueous phases with different pH. In method 1, the peak appeared broad, with tailing and fair intensity(Appendix 1.1). While method 2 showed higher peak intensity with less tailing and the resolution is better (Appendix 1.2). In method 3 is the selected the peak showed no tailing, good resolution and with better intensity.

1.1.3. Liquid-liquid extraction

The liquid-liquid extraction using no buffer and methyl tertiary butyl ether had shown the higher intensity, better resolution with no matrix effect and higher recovery.

1.1.4. Choice of internal standard

Propranolol and Bisoprolol were tested for internal standard.

For Propranolol, the chromatogram showed 2 peaks m/z=260.15 and m/z=183.15.

The daughter peak chosen had shown a good intensity, with no tailing and good resolution but the retention time is high.

For Bisoprolol, the chromatogram showed 2 peaks: m/z= 326.1 and m/z= 115.9.

The daughter peak had shown a good intensity, with no tailing and good resolution, in addition that the retention time was in the same range of that of pethidine.

Thus, Bisoprolol was chosen as an internal standard for the Pethidine analysis in LC-MS/MS.

1.1.5. Adjusting the retention time

After using an organic phase composed of 80% Methanol, and the flow rate was increased to 0.3ml/min, the retention time of Pethidine and Bisoprolol were 0.46 minutes and 0.45 minutes respectively (Appendix 1.5).

1.2 Validation

The validation of the method of analysis that had been accredited during the study was evaluated in terms of sensitivity, stability, recovery, linearity, intra-day and inter-day precision and accuracy.

1.2.1. Sensitivity

The sensitivity evaluates that the efficiency of the method of analysis can detect the lower limit of quantification (LLOQ) that was determined to be 20 ng/ml. Ten different samples of 20 ng/ml were injected into the LC-MS/MS. The measured

concentration was back calculated from the calibration curve of day 1. The results were tabulated and the accuracy and precision were determined (table 3.1.1).

The accuracy and precision of the LLOQ should not exceed 20%. The maximum accuracy deviation was 19.88% <20%., with an average of 8.87 %. Consequently, the method of analysis accredited during pethidine determination study was accurate in terms of sensitivity.

The precision calculated was equal to 7.294 <20%, thus the method was precise in terms of sensitivity.

Table 3.1.1: Validation in term of sensitivity

Sample No.	AUC Drug	AUC IS	Ratios	Measured Conc.	Precision %	Accuracy %
1	948746.043	24642598.943	0.039	21.847		109.24
2	848406.842	21644930.278	0.039	22.263		111.32
3	899631.067	22100168.719	0.041	23.165		115.83
4	875528.394	22455364.568	0.039	22.140		110.70
5	821883.627	20658742.908	0.040	22.614		113.07
6	830839.120	23996700.222	0.035	19.532		97.66
7	843660.165	21445558.097	0.039	22.349		111.74
8	772712.915	23168502.765	0.033	18.773	**7.294**	93.86
9	910121.580	21637063.534	0.042	23.975		119.88
10	780128.086	20953772.671	0.037	21.089		105.45
Mean	**853165.784**	**22270340.271**	**0.038**	**21.775**		**108.87**
STD	**56209.810**	**1303024.564**	**0.003**	**1.588**		**7.94**
CV%	**6.588**	**5.851**	**6.929**	**7.294**		**7.29**
Min.	**772712.915**	**20658742.908**	**0.033**	**18.773**		**93.86**
Max.	**948746.043**	**24642598.943**	**0.042**	**23.975**		**119.88**

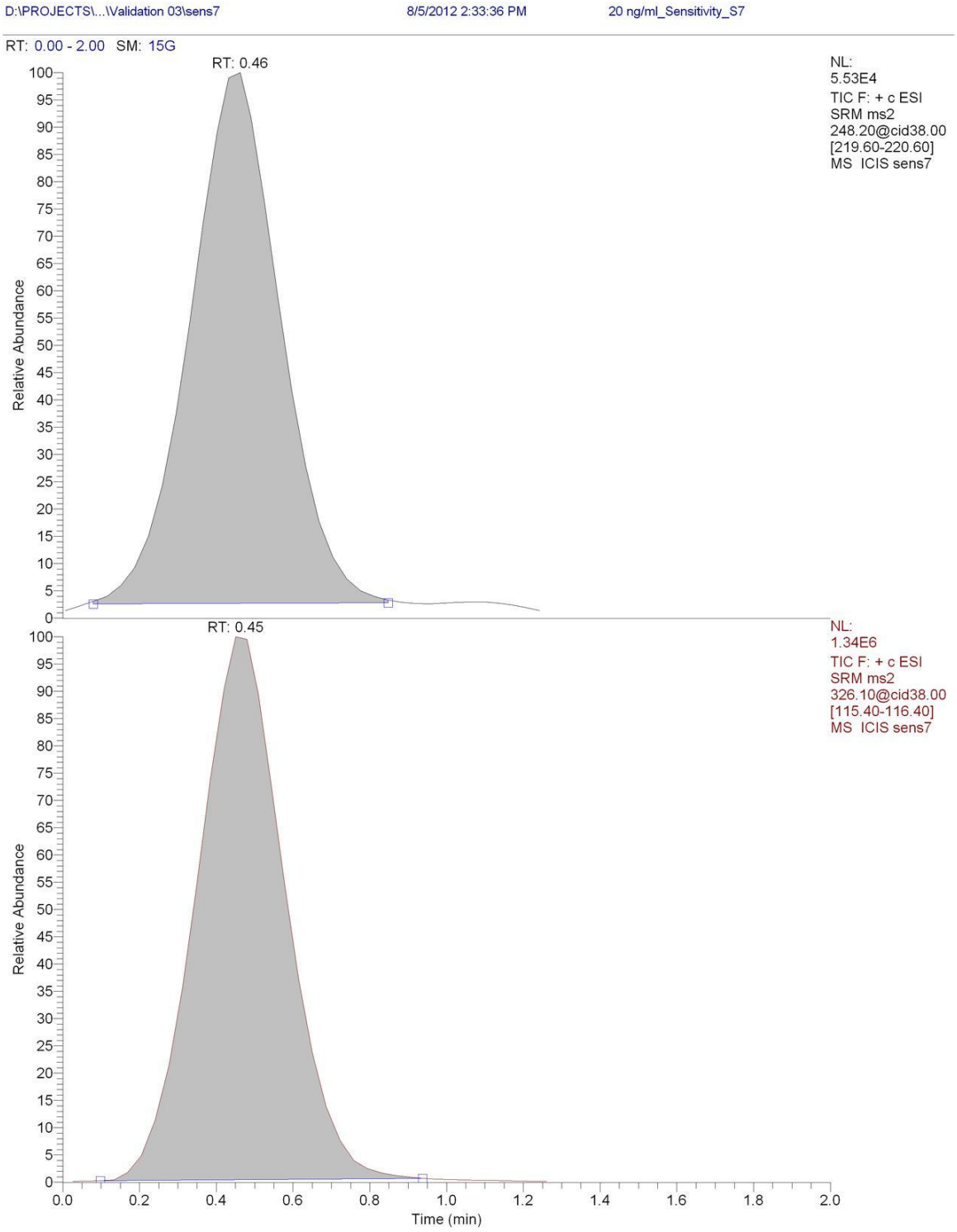


Figure3.1: Chromatogram of the LLOQ of Pethidine (above) and internal standard (below).

1.2.2. Stability

The validation of the stability had evaluated the autosampler stability, bench top stability and 3 freeze and thaw cycles

1.2.2.1. Autosampler stability

The autosampler stability validation ensures the stability of Pethidine and the internal standard over 24 hours. The results shown in table 3.1.2, prove that the Pethidine is stable at the low QC with a stability of 90.19%, and a maximum accuracy deviation of 4.33 % <15%. The mid QC autosampler stability, table 3.1.3, showed a stability of 99.97% and a maximum accuracy deviation of 11.99 %< 15%. The high QC autosampler stability, table 3.1.4, had a value of 93.48 % with a maximum accuracy deviation of 3.97%.

The method of analysis, the containers used during analysis and the medium are compatible with Pethidine ensuring its stability for 24 hours at 15°C on the autosampler.

Table3.1.2: Autosampler stability for low QC = 60 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy %	Stability
0.00 Hour	2161281	20087871	0.108	63.112	64.876	105.19	100.00
	2400844	21441312	0.112	65.728		109.55	
	2516956	22458032	0.112	65.789		109.65	
24.00 Hours	1982719	20434065	0.097	56.804	58.509	94.67	90.19
	2124816	20556554	0.103	60.587		100.98	
	1954805	19694004	0.099	58.135		96.89	

Table3.1.3: Autosampler stability for mid QC = 500 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy %	Stability
0.00 Hour	19261834	21005306	0.917	546.526	541.433	109.31	100.00
	18779285	21287571	0.882	525.726		105.15	
	19677027	21243893	0.926	552.048		110.41	
24.00 Hours	18717193	19923157	0.939	559.946	541.257	111.99	99.97
	17835241	19600445	0.910	542.311		108.46	
	18813038	21497688	0.875	521.514		104.30	

Table3.1.4: Autosampler stability for high QC = 800 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy %	Stability
0.00 Hour	30112346	20270995	1.485	886.054	840.583	110.76	100.00
	27813061	20771016	1.339	798.583		99.82	
	27466062	19569076	1.404	837.113		104.64	
24.00 Hours	25024628	19417068	1.289	768.580	785.805	96.07	93.48
	28121302	20438466	1.376	820.603		102.58	
	27728777	21524992	1.288	768.232		96.03	

1.2.2.2. Bench top stability

This validation study was performed over 6 hours in order to ensure that Pethidine is stable for this period stored on the bench at 25°C, taking in consideration the method of extraction, medium used for reconstitution and the container system.

This validation study was performed on the QC concentrations. The QC low validation had proven a stability of 93.84% and a maximum accuracy deviation of 3.79% (table 3.1.5). And the QC mid stability had a value of 96.39%, with an accuracy deviation of maximum of 4.92 % (table 3.1.6). And the QC high had shown a stability of 96.63% and a maximal accuracy deviation of 4.67% (table 3.1.7).

Pethidine was consequently considered stable for 6 hours after the extraction and reconstitution procedures used in the method of analysis.

Table3.1.5: Bench top stability for low QC = 60 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy	Stability
0.00 Hour	1983716	20887090	0.095	58.347	63.378	97.25	100.00
	2364207	21613840	0.109	66.398		110.66	
	2308874	21462797	0.108	65.388		108.98	
6.00 Hours	2056168	20655465	0.100	60.902	59.472	101.50	93.84
	2276015	23331811	0.098	59.787		99.64	
	1917591	20429432	0.094	57.728		96.21	

Table3.1.6: Bench top stability for mid QC = 500 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy %	Stability
0.00 Hour	19105442	21213833	0.901	508.447	513.780	101.69	100.00
	17747479	18675832	0.950	536.202		107.24	
	18302520	20808588	0.880	496.689		99.34	
6.00 Hours	18274062	21716689	0.841	475.408	495.207	95.08	96.39
	18254007	19763487	0.924	521.303		104.26	
	17479565	20192628	0.866	488.909		97.78	

Table3.1.7: Bench top stability for high QC = 800 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy %	Stability
0.00 Hour	25954729	17649474	1.471	826.873	811.115	103.36	100.00
	29297182	19989105	1.466	824.131		103.02	
	26616757	19136973	1.391	782.340		97.79	
6.00 Hours	26154484	19293232	1.356	762.660	783.768	95.33	96.63
	27291661	18949226	1.440	809.937		101.24	
	25880238	18694807	1.384	778.708		97.34	

1.2.2.3. Freeze-thaw stability

The freeze thaw stability study ensures that after 3 cycles of thawing and re-freezing, pethidine would still be stable and determined accurately by the LC-MS/MS.

This study was also performed on the QC concentrations only. The QC low analysis showed after 3 freeze and thaw cycles, a 96.48 % stability and a maximal accuracy deviation of 4.19 (table 3.1.8). Whereas, the QC mid analysis revealed a 100.75% stability after 72 hours and an accuracy deviation limited to 2.46 % (table 3.1.9). And finally, the QC high analysis showed 97.05 % stability with a maximal accuracy deviation of 7.79% (table 3.1.10).

The freeze-thaw cycle represents the worst case scenario that the sample may pass through during analysis. And the method of analysis which has been accredited showed to be valid in terms of stability especially, in the freeze-thaw stability.

Table3.1.8: Freeze-thaw stability for QC low = 60 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy	Stability
0.00 Hour	1983716	20887090	0.095	58.347	63.378	97.25	100.00
	2364207	21613840	0.109	66.398		110.66	
	2308874	21462797	0.108	65.388		108.98	
72.00 Hour	2130164	20795252	0.102	62.516	61.150	104.19	96.48
	2058007	21218723	0.097	59.474		99.12	
	1934321	19238786	0.101	61.459		102.43	

Table3.1.9: Freeze-thaw stability for QC mid = 500 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy %	Stability
0.00 Hour	19105442	21213833	0.901	508.447	513.780	101.69	100.00
	17747479	18675832	0.950	536.202		107.24	
	18302520	20808588	0.880	496.689		99.34	
72.00 Hour	18409220	18663557	0.986	556.360	517.620	111.27	100.75
	17878802	20705374	0.863	487.705		97.54	
	18235540	20233985	0.901	508.794		101.76	

Table 3.1.10: Freeze-thaw stability for QC high = 800 ng/ml

Time	AUC Drug	AUC IS	Ratios	Measured Conc.	Mean Measured	Accuracy	Stability
0.00 Hour	25954729	17649474	1.471	826.873	811.115	103.36	100.00
	29297182	19989105	1.466	824.131		103.02	
	26616757	19136973	1.391	782.340		97.79	
72.00 Hour	25767719	19657259	1.311	737.642	787.165	92.21	97.05
	26484486	18436381	1.437	807.860		100.98	
	28357167	19541942	1.451	815.993		102.00	

1.2.3. Precision and accuracy

The validation of the method of analysis also includes the evaluation of the results variation of 10 samples of each QC concentration injected in the same day; this is called intra-day variation. In addition, it evaluates the results variation between 10 samples of each QC concentration injected on 2 different days; this was called the inter-day validation. The accuracy and precision of every analytical day were calculated, and they should not exceed 15% variation. A calibration curve was plotted for every process.

1.2.3.1. Intra-day validation

10 samples of every QC concentration were prepared and injected into the LC-MS/MS, the results are shown in the table 3.1.11.

The mean accuracy of the QC low=60 ng/ml was equal to 106.01% with a maximal deviation of 11.69%, and the precision was equal to 4.536%.

The mean accuracy of the QC mid=500ng/ ml was equal to 104.21% with a maximal deviation of 11.92%, and the calculated precision had a value of 5.347 %.

The mean accuracy of the QC high=800ng/ ml was equal to 100.22% (almost 100%) with a maximal variation of 7.92%, with a precision of 5.239%.

All the accuracy and precision values were less than 15%, which ensures that the method of analysis chosen is accurate and precise if the analysis has been repeated during the same day.

Table3.1.11: The accuracy and precision of the QC concentrations on day 1

Theo. Conc.	AUC Drug	AUC IS	Ratios	Measured Conc.	Precision %	Accuracy %
60 ng/ml QC Low	2253022	19782734	0.114	66.873	4.536	111.45
	2232996	19565848	0.114	67.015		111.69
	2064480	18536750	0.111	65.370		108.95
	2191929	19500336	0.112	65.986		109.98
	2263879	21772530	0.104	60.954		101.59
	1986094	19198616	0.103	60.638		101.06
	2390927	22233313	0.108	63.080		105.13
	2185166	19684348	0.111	65.154		108.59
	1937387	19312561	0.100	58.767		97.95
	2238528	21097087	0.106	62.225		103.71
Mean	**2174440.822**	**20068412.232**	**0.108**	**63.606**		**106.01**
STD	**138547.244**	**1207717.227**	**0.005**	**2.885**		**4.81**
CV%	**6.372**	**6.018**	**4.455**	**4.536**		**4.54**
500 ng/ml QC Mid	17424221	18557919	0.939	559.613	5.437	111.92
	16514075	19198129	0.860	512.598		102.52
	16946378	19362827	0.875	521.563		104.31
	16233636	20923155	0.776	462.237		92.45
	17376456	20996364	0.828	493.129		98.63
	19769421	21602951	0.915	545.408		109.08
	18268557	20337922	0.898	535.329		107.07
	19126413	21268891	0.899	535.936		107.19
	18773567	22019089	0.853	508.067		101.61
	19450192	21608527	0.900	536.443		107.29
Mean	**17988291.533**	**20587577.471**	**0.874**	**521.032**		**104.21**
STD	**1261847.663**	**1177514.888**	**0.047**	**28.327**		**5.67**
CV%	**7.015**	**5.720**	**5.425**	**5.437**		**5.44**
800 ng/ml QC High	26931110	21509678	1.252	746.631	5.239	93.33
	27612741	19721760	1.400	835.065		104.38
	26340796	20711838	1.272	758.414		94.80
	28237424	20627996	1.369	816.415		102.05
	26708964	18702584	1.428	851.772		106.47
	28065221	19388554	1.448	863.374		107.92
	26725897	20962713	1.275	760.296		95.04
	27212483	19754097	1.378	821.595		102.70
	27275971	20409746	1.336	797.023		99.63
	25127793	19535251	1.286	767.077		95.88
Mean	**27023839.977**	**20132421.659**	**1.344**	**801.766**		**100.22**
STD	**898225.429**	**850529.506**	**0.070**	**42.002**		**5.25**
CV%	**3.324**	**4.225**	**5.231**	**5.239**		**5.24**

1.2.3.2. Inter-day validation

Injection of 10 different samples from each QC concentration on the second day was performed and the results are shown in the table 3.1.12.

The mean accuracy and the precision of each concentration had not exceeded 15%. The accuracy values were 98.81%, 98.46% and 96.89% for the QC low, mid and high respectively. Whereas, the precision values were 7.645%, 4.254% and 4.020% for the QC low, mid and high respectively.

But, in order to evaluate the variations between day 1 and day 2, the accuracy and precision of the 20 samples of each QC concentration were calculated.

The mean accuracy values were equal to 102.41%, 101.333% and 98.555% for the QC low, mid and high respectively.

The values of the precision were 7.016%, 5.6%, and 4.883 % for the QC low, mid and high respectively.

Consequently, according to ICH guidelines, the results are less than 15%which means that the method is valid in inter-day analysis.

Table 3.1.12: The accuracy and precision of the QC concentrations on day 2

Theo. Conc.	AUC Drug	AUC IS	Ratios	Measured Conc.	Precision %	Accuracy %
60 ng/ml QC Low	2307773	23048402	0.100	64.345	7.645	107.24
	2286518	26048531	0.088	57.159		95.26
	2113866	21602709	0.098	63.021		105.04
	2603228	27103415	0.096	61.971		103.29
	1606135	20586386	0.078	51.479		85.80
	2376706	23009182	0.103	66.188		110.31
	2237845	26227858	0.085	55.730		92.88
	2108152	24415700	0.086	56.324		93.87
	2107943	23903089	0.088	57.396		95.66
	2152897	23566634	0.091	59.239		98.73
Mean	**2190106.168**	**23951190.602**	**0.091**	**59.285**		**98.81**
STD	**256600.781**	**2066242.279**	**0.008**	**4.532**		**7.55**
CV%	**11.716**	**8.627**	**8.517**	**7.645**		**7.64**
500 ng/ml QC Mid	19954785	23391843	0.853	502.537	4.254	100.51
	18409845	21848898	0.843	496.445		99.29
	20087276	24365133	0.824	485.870		97.17
	19411667	23865138	0.813	479.447		95.89
	20132180	23170684	0.869	511.731		102.35
	18621641	21012988	0.886	521.818		104.36
	18180797	21884748	0.831	489.551		97.91
	20110138	23884878	0.842	496.074		99.21
	19253795	22890869	0.841	495.580		99.12
	17422017	23154864	0.752	443.959		88.79
Mean	**19158414.136**	**22947004.254**	**0.835**	**492.301**		**98.46**
STD	**957050.709**	**1059485.230**	**0.036**	**20.941**		**4.19**
CV%	**4.995**	**4.617**	**4.307**	**4.254**		**4.25**
800 ng/ml QC High	32139627	24063132	1.336	783.382	4.020	97.92
	31507672	24280786	1.298	761.267		95.16
	32557842	23667117	1.376	806.672		100.83
	28531643	22893185	1.246	731.386		91.42
	33883081	24132114	1.404	823.205		102.90
	30690451	23889002	1.285	753.743		94.22
	32238829	24817518	1.299	762.080		95.26
	33103594	25016322	1.323	776.190		97.02
	29620901	23476344	1.262	740.372		92.55
	31467629	22700153	1.386	812.825		101.60
Mean	**31574126.911**	**23893567.252**	**1.321**	**775.112**		**96.89**
STD	**1607907.369**	**745373.531**	**0.054**	**31.159**		**3.89**
CV%	**5.092**	**3.120**	**4.052**	**4.020**		**4.02**

1.2.4. Calibration curve and linearity

In order to evaluate the linearity of the calibration curve, the latter was plotted in 3 different days, the correlation factor (R^2) is calculated, for each. The mean of the three ratios (AUC of Pethidine/AUC of internal standard) was then plotted against the theoretical concentration ranging from 20 ng/ml to 1000 ng/ml.

1.2.4.2. Day 1 calibration curve

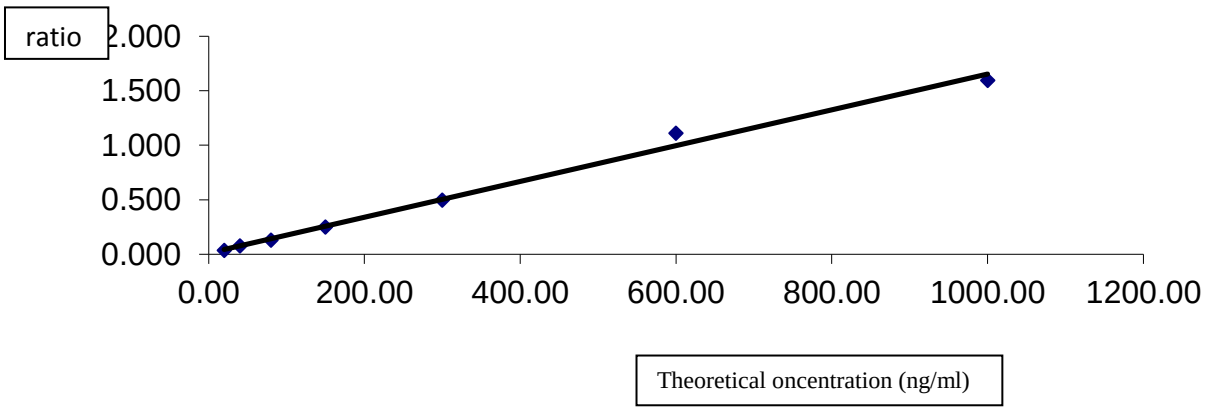
The calibration curve for the first day was plotted and considered valid, as the maximum deviation of the accuracy was 11.99 % and the LLOQ accuracy was equal to 92.12% which is below 20% as shown in table 3.1.13.

The curve was linear as shown in figure 3.1.1 with a regression equation Y=0.00946342+0.00178991*X; and R^2 = 0.9968.

Table 3.1.13: Measured concentrations and accuracy for the calibration curve of day 1

Theoretical conc. ng/ml	Drug Area	IS Area	Ratio	Measured Conc. (ng/ml)	Accuracy (%)
20.00	475307	20215053	0.024	18.423	92.12
40.00	1492957	21110835	0.071	44.797	111.99
80.00	2340993	15779174	0.148	88.174	110.22
150.00	4614862	19903520	0.232	134.825	89.88
300.00	8427181	16848393	0.500	284.729	94.91
600.00	19602216	18903488	1.037	584.625	97.44
1000.00	31294500	16988784	1.842	1034.426	103.44

correllation	slope	factor	RSQ	Intercept
0.998399	**0.001790**	**558.687308**	**0.996800**	**-0.009463**



Y = -0.00946342+0.00178991*X R^2 = 0.9968

Figure3.1.1: Calibration curve of day 1

1.2.4.3. Day 2 calibration curve

On day 2, the calibration curve had also been plotted, with a maximum accuracy deviation of 10.2%, and the accuracy deviation of the LLOQ= 7.63 %< 15%, consequently the curve was considered valid (table 3.1.14).

The curve was linear and with the following regression equation (figure 3.1.2):

$Y = 0.00191995+0.00167436*X$, and $R^2 = 0.9946$.

1.2.4.4. Day 3 calibration curve

On day 2, the calibration curve had also been plotted, with a maximum accuracy deviation of 13.83%, and the accuracy deviation of the LLOQ= 4.14 %< 15%, consequently the curve was considered valid (table 3.1.15).

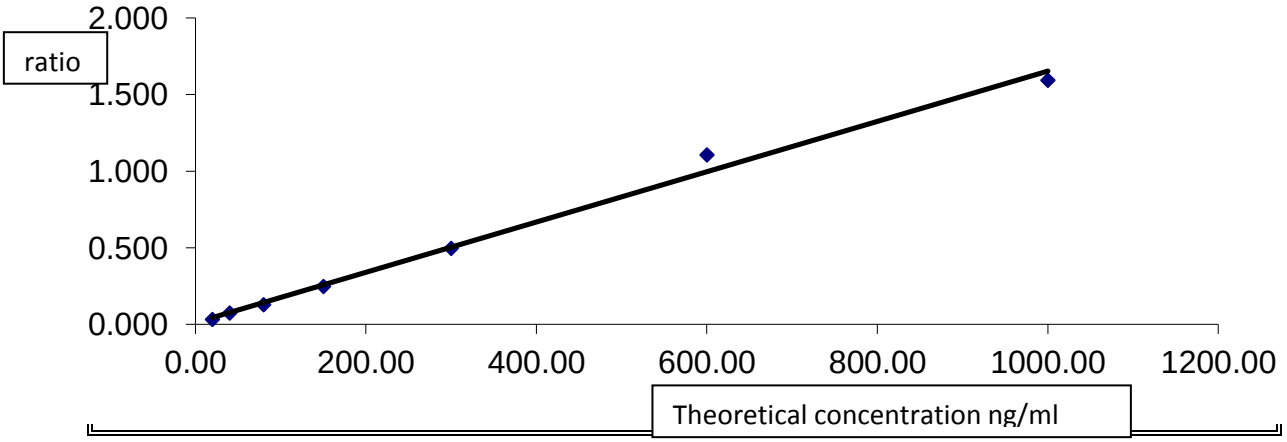
The curve was linear and with the following regression equation (figure 3.1.3):

$Y = -0.0104363+0.00171829*X$, and $R^2 = 0.9973$

Table3.1.14: Measured concentration and accuracy for the calibration curve of day 2

Theoretical conc. ng/ml	Drug Area	IS Area	Ratio	Measured Conc.	Accuracy
20.00	765429	22519457	0.034	19.153	95.77
40.00	1767052	23768014	0.074	43.256	108.14
80.00	2750684	21323719	0.129	75.896	94.87
150.00	5276809	21287668	0.248	146.899	97.93
300.00	10519300	21223499	0.496	294.874	98.29
600.00	21551909	19465838	1.107	660.102	110.02
1000.00	30945819	19435215	1.592	949.819	94.98

correllation	slope	factor	RSQ	Intercept
0.997296	**0.001674**	**597.243126**	**0.994600**	**0.001920**



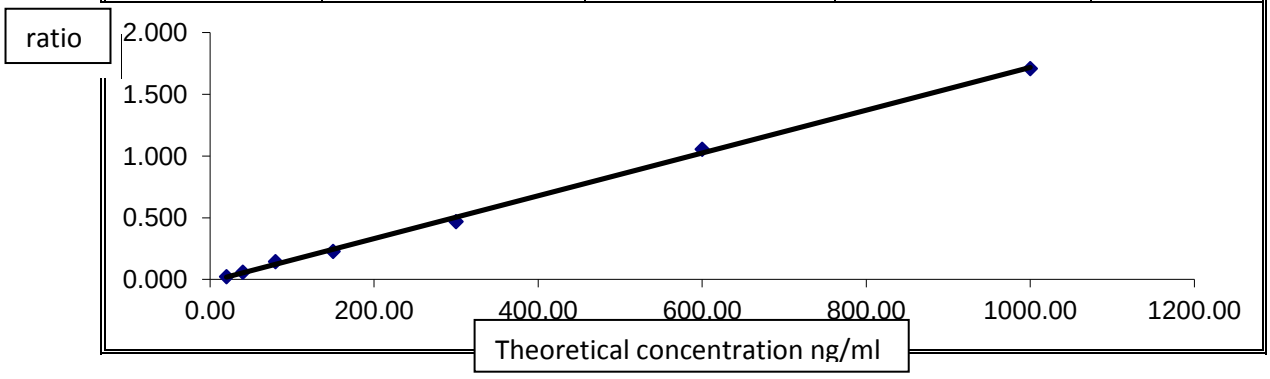
Equation **Y = 0.00191995+0.00167436*X R^2 = 0.9946**

Figure:3.1.2: Calibration curve of day 2

Table3.1.15: Measured concentration and accuracy of the calibration curve of day 3

Theoretical conc. ng/ml	Drug Area	IS Area	Ratio	Measured Conc. (ng/ ml)	Accuracy (%)
20.00	396582	18094811	0.022	18.829	94.14
40.00	1256360	20791609	0.060	41.240	103.10
80.00	2484637	17013369	0.146	91.065	113.83
150.00	4125326	18121045	0.228	138.563	92.38
300.00	8879746	18921068	0.469	279.197	93.07
600.00	18098356	17140103	1.056	620.586	103.43
1000.00	32093509	18781939	1.709	1000.520	100.05

correllation	slope	factor	RSQ	Intercept
0.998649	**0.001718**	**581.973939**	**0.997300**	**-0.010436**



Equation **Y = -0.0104363+0.00171829*X R^2 = 0.9973**

Figure 3.1.3: Calibration curve of day 3

In order to evaluate the reproducibility and the linearity of the calibration curve, the mean of the 3 ratios of the previous calibration curve were plotted against the theoretical concentrations raging from 20 ng/ ml to 1000 ng/ ml (figure 3.1.16).

The resulted maximal deviation of the accuracy was 10.54 % which is less than 15% and the LLOQ accuracy deviation is 0.04% which is below 20%. The calibration curve was considered then valid.

On the other hand, the calibration curve was found to be linear with a regression equation (figure 3.1.4)

$Y= - 0.008214 + 0.001735* X$, and $R^2 = 0.999039$

Table 3.1.16: Measured concentrations and accuracy of the overall calibration curve

Theoretical concentration (ng/ ml).	Ratio 1	Ratio 2	Ratio3	Mean ratio	Measured concentration (ng/ml)	Accuracy %

20.000	0.024	0.034	0.022	0.026	19.993	99.96
40.000	0.071	0.074	0.060	0.068	44.214	110.54
80.000	0.148	0.129	0.146	0.141	86.078	107.60
150.000	0.232	0.248	0.228	0.236	140.641	93.76
300.000	0.500	0.496	0.469	0.488	286.219	95.41
600.000	1.037	1.107	1.056	1.067	619.535	103.26
1000.000	1.842	1.592	1.709	1.714	992.835	99.28

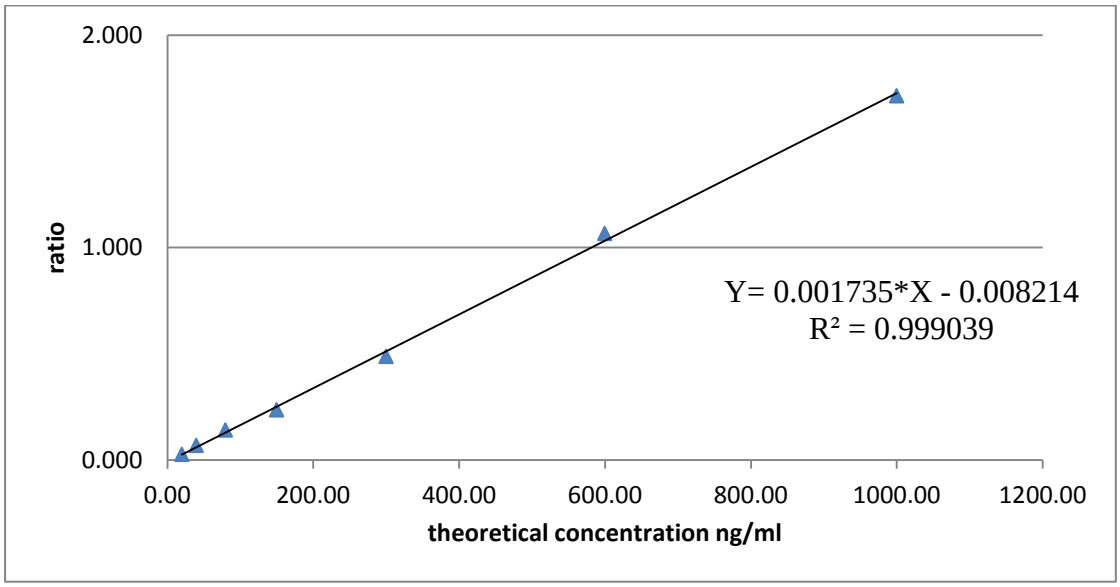


Figure 3.1.4: Overall calibration curve

1.2.5. Recovery and matrix effect

The efficiency of the extraction method is expressed by the recovery where as the effect of the plasma of the efficiency of the method of analysis is expressed by the matrix effect.

In order to evaluate the recovery and matrix effect, 3 different samples of each QC concentrations were injected. The first sample was a Pethidine solution (table 3.1.17) the second was Pethidine in plasma where Pethidine was added before the extraction procedure (table 3.1.18), and the third was a Pethidine spiked plasma where pethidine was added just before injection (table 3.1.19).

The Coefficient of variation of three different reading for each sample was calculated.

The recovery is the comparison between mean AUC in plasma and mean AUC in spiked plasma.

The recovery of the Pethidine was 80.61%, 87.08% and 94.56% for the QC low, mid and high respectively (table 3.1.20), whereas the recovery of the internal standard was 85.05%, 98.12% and 99.99& for the QC low, mid and high respectively (table 3.1.21).

The matrix effect on Pethidine was 94.86%, 104.79% and 101.25% for the QC low, mid and high respectively (table 3.1.22), whereas the matrix effect on the internal standard was 94%, 86.55% and 85.95% for the QC low, mid and high respectively (table 3.1.23).

Table3.1.17: Mean AUC of Pethidine and IS and corresponding C.V. for pethidine solution

Concentration	AUC Drug	AUC IS	Mean Drug	C.V % (Drug)	Mean I.S	C.V % (I.S)
60.00ng/ml	3207527	32998152	3279376.714	1.898	31310974.532	4.667
QC Low	3316647	30453392				
	3313957	30481380				
500.00ng/ml	21704485	27901156	22283876.952	3.646	27923365.517	3.327
QC Mid	23212489	27005740				
	21934657	28863200				
800.00ng/ml	35958628	26100933	33587831.667	6.615	25142744.041	3.543
QC High	33251607	24339811				
	31553260	24987488				

Table3.1.18: Mean AUC of Pethidine and IS and corresponding C.V. in plasma (spiked before extraction)

Concentration	AUC Drug	AUC IS	Mean Drug	C.V % (Drug)	Mean I.S	C.V % (I.S)
60.00ng/ml	2621169	25026904	2507466.727	4.661	25033939.870	0.364
QC Low	2513571	24946637				
	2387660	25128279				
500.00ng/ml	19790179	24777023	20333336.523	5.432	23712096.799	6.192
QC Mid	21604334	24322039				
	19605497	22037228				
800.00ng/ml	31678388	21967863	32155693.243	2.177	21609045.585	1.440
QC High	32959313	21444630				
	31829379	21414643				

Table3.1.19: Mean AUC of Pethidine and IS and corresponding C.V. in spiked plasma

Concentration	**AUC Drug**	**AUC IS**	**Mean Drug**	**C.V % (Drug)**	**Mean I.S**	**C.V %(I.S)**
60.00ng/ml	3292846	31511273	3110806.453	5.076	29433499.175	8.306
QC Low	3028658	26739568				
	3010916	30049656				
500.00 ng/ml	21853083	23651730	23351065.592	8.527	24167014.954	2.074
QC Mid	25610536	24196392				
	22589578	24652923				
800.00ng/ml	37346818	22656064	34006148.182	9.756	21610732.861	8.188
QC High	30712008	19567809				
	33959619	22608325				

Table 3.1.20: Absolute recovery for Pethidine

Concentration	Mean Plasma	Mean Spiked	Absolute Recovery (%)
60 ng/ml QC Low	2507466.727	3110806.453	80.61
500 ng/ml QC Mid	20333336.523	23351065.592	87.08
800ng/ml QC High	32155693.243	34006148.182	94.56

Table 3.1.21: Absolute recovery for IS

Concentration	Mean Plasma	Mean Spiked	Absolute Recovery (%)
60 ng/ml QC Low	25033939.870	29433499.175	85.05
500 ng/ml QC Mid	23712096.799	24167014.954	98.12
800ng/ml QC High	21609045.585	21610732.861	99.99

Table 3.1.22: Matrix effect for Pethidine

Concentration	Mean Spiked	Mean Solution	Matrix Effect (%)
60 ng/ml QC Low	3110806.453	3279376.714	94.86
500 ng/ml QC Mid	23351065.59	22283876.95	104.79
800ng/ml QC High	34006148.18	33587831.67	101.25

Table 3.1.23: Matrix effect for IS

Concentration	Mean Spiked	Mean Solution	Matrix Effect (%)
60 ng/ml QC Low	29433499.175	31310974.532	94
500 ng/ml QC Mid	24167014.954	27923365.517	86.55
800ng/ml QC High	21610732.861	25142744.041	85.95

2. Clinical Part

2.1. Maternal baseline characters

Following a single injection of 50mg intravenously, blood sampling was performed at different intervals of time. The concentrations were measured at 0, 120 minutes and just after delivery. 15 subjects were included in the study of whom 12 (80%) have completed the study and 3 (20%) were withdrawn. In fact the withdrawn cases were transferred to the operation room for Caesarean section.

The baseline characters of the subjects in labor are shown in the table 3.2.1

Subject	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Age (years)	27	36	34	28	34	27	17	24	16	26	20	33	24	30	18
weight KG	86	90	75	78	74	71	73	70	68	73	69	84	72	86	65
completion	c	c	c	w	c	c	c	c	c	w	c	w	c	c	c

Table 3.2.1: Baseline characters of subjects in labor, (c) stands for completion of the study and (w) stands for withdrawal from the study

The mean age was 26.26 years and the mean weight was 75.6 Kg.

All the subjects have completed 36 weeks gestation on the admission to the delivery room. All the mothers were healthy and non smokers except subject “10” and “12”. In fact, according to the subject history taken whether from the subject file or upon questioning, none of the 13 subject had experienced any chronic disease or taking chronically any medication. Nevertheless, woman “10” had preeclampsia and gestational diabetes. She had being treated with methyldopa for lowering hypertension but she was not on any pharmacological treatment for diabetes. The blood sample from this patient was taken at 0 time, but as a result of fetal distress and the patient felt very dizzy, she was transferred for Caesarian section.

In addition, subject “12” had experienced gestational diabetes, and she was on low sugar diet without being treated pharmacologically.

Subject “4” has been withdrawn in less than 30 minutes of pethidine injection. In actual fact, there was a severe drop of fetal heart rate and neonatal depression. The first sample for this subject was taken 5 minutes after administration instead of 0 time. The analytical determination of Pethidine in plasma has demonstrated a very high concentration, (42,017.869 ng/ ml) after 5 minutes. This concentration exceeds the maximum concentration (Miskolczi P, Vereczkey L.,1985). The amount of pethidine transferred to the fetus has been strongly correlated with dosage and the mother’s drug metabolic rate (Morselli PL, Rovei V., 1982).

Neonatal depression which developed in subject “4” could be secondary to the exceedingly high level of Pethidine in the mother’s blood. This might be subsequent to a very low, almost non-existent, Pethidine metabolism which had resulted in a high amount of Pethidine transferred to the fetus and probably caused a very rapid decrease in fetal heart rate. The inter-patient genetic variability in drug metabolism might be a potentially important factor that should be further investigated.

2.2. Maternal pharmacokinetics

The plasma Pethidine concentrations had been determined in the subjects who had completed the study by LC-MS/MS according to the validated method described before. The results are summarized in the table 3.2.2.

Table 3.2.2:Pethidine concentrations (ng/ml) at different samplings in women in labor

Time interval Subject	0	5	30	60	75	90	120	140	160	180	213
M1	0					257.028	233.445				
M2	139.013		199.988				116.429				
M3	0						158.319			126.511	
M4		42,017.87									
M5	0						208.56				304.299
M6	0			129.25			257.538				
M7	0				110.656		88.341				
M8	0									99.896	
M9	0				268.069		215.113				
M10	0										
M11	0						217.572	136.83			
M12	0		331.228				413.014				
M13	0					355.867	133.503				
M14	0								109.15		
M15	0					266.243	196.038				

Almost all the samples had been withdrawn at 0 and 120 minutes. However, in subject 3, the blood sample has been taken 1 minute after Pethidine injection from a different site of the injection. And subject 12 (this case was withdrawn), the 0 time sample was missed, so the first sample was taken 30 minutes after administration.

The cases where the blood sampling occurs between 0 and 120 minutes correspond also to the post delivery sample.

Regarding cases no 8 and 14, blood sampling could not be taken at 120 minutes because the subjects were in the delivery room by this time, and the sample could not be collected immediately.

Regarding case no 2, this was the only case where Pethidine dose was given at 2 hours prior to the withdrawal of blood sample at time 0. This explains the concentration 139.013 ng/ml at the pre-administration time.

The concentrations that were below the lower limit of quantification (20ng/ml) determined by the validated method of analysis, were considered null during the statistical analysis.

The mean of maternal concentration at 120 minutes was equal to 196.1027 ng/ ml and the median was 196.038 ng/ ml.

The case no 12 has the highest concentration (413.014 ng/ml) at 120 minutes, this may have resulted in the fetal distress and the transfer of the mother to the operation room for Caesarean section delivery.

It was found that after an intravenous Pethidine injection, the mean of $T_{1/2}^{ß}$ is 157 minutes and the maximum concentration is reached within the first 30 minutes (around 800 ng/ml after 100 mg IV bolus injection) (Husemeyer RP, *et al*., 1982). Nevertheless, among the subjects who had completed the study, 2 cases (16.7 %) had shown a different profile than the normal intravenous profile. The plasma Pethidine concentration has been increasing by time. In case no5, at 120 minutes the calculated concentration was 208.56 ng/ ml, and at 213 minutes the concentration was 304.299 ng/ ml. In case no 6, the concentration was 129.25 ng/ml at 62 minutes and at 120 minutes, 257.538 ng/ ml. There are 2 possible explanations for these profiles. The increase in the plasma concentration may be due to a very slow drug metabolism which may be caused by different factors such as smoking (Santos AC, *et al*., 2007).

2.3. Newborn baseline characters

The baseline characters of the newborn included the newborn weight, sex and the Apgar score. The data distribution is shown in the table 3.2.3.

Table 3.2.3: Baseline characters of the newborn related to the cases that completed the study

	1	2	3	5	6	7	8	9	11	13	14	15
Sex of the newborn	M	M	M	M	F	M	F	M	M	F	F	F
Weight of KG	3.4	3.7	3.2	2.9	3.3	2.8	2.88	2.8	3.4	3.8	3.45	2.9
Apgar score at 1 min	8	7	7	8	8	7	7	8	8	9	8	9

The mean weight of the newborns was 3.21 kg, and 42% of the newborn were female and 58% were male. The mean Apgar score was 8, all the scores were above or equal to 7 which did not require any antidote to Pethidine (naloxone) or medical assistance.

2.4. Newborn pharmacokinetics

Since the plasma Pethidine concentration withdrawn from the umbilical cord reflects the plasma concentration in the newborn, it was decided to take blood from the cord rather than to withdraw from the newborn. The plasma Pethidine concentration (ng/ml) for the 12 newborns at the time of delivery are shown in the figure 3.2.1 and table 3.2.4.

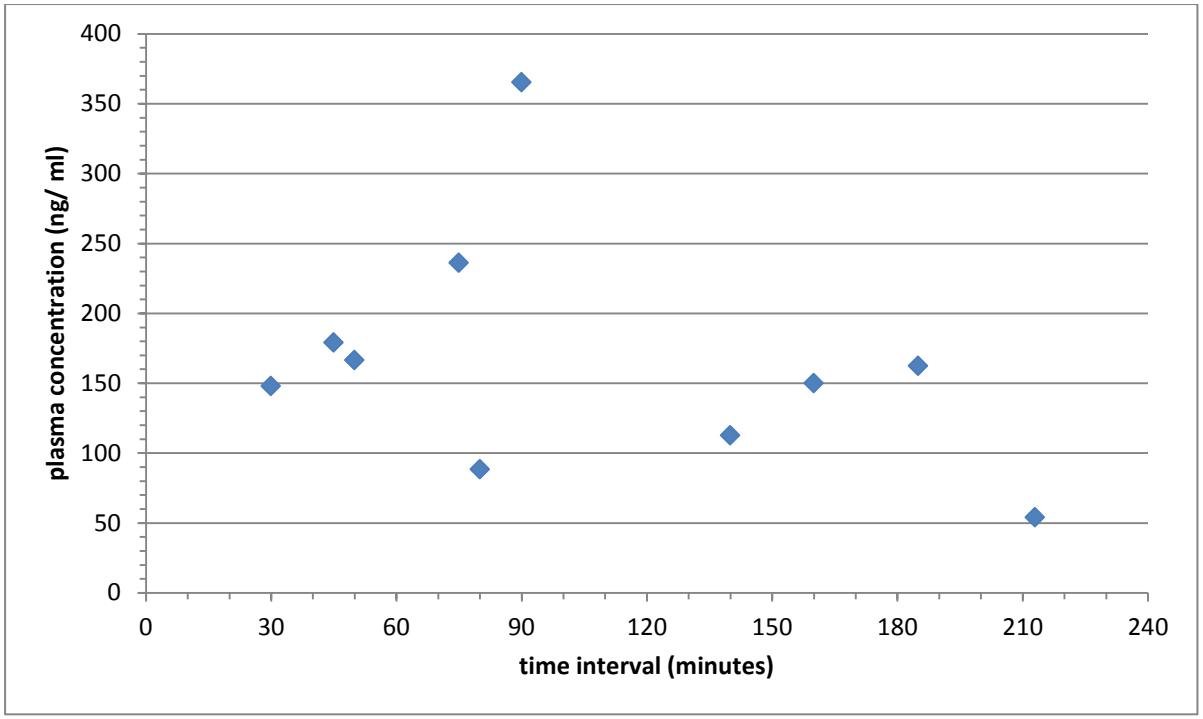


Figure 3.2.1: Newborn plasma concentration at time of delivery (time 0 represents the time of Pethidine administration to the mother)

Table 3.2.4: The Pethidine concentration ng/ml in umbilical cord and time of the sampling after injection

	time interval (minutes)									
	30	45	50	75	80	90	140	160	185	213
b1						365.167				
b2	147.822									
b3									99.996	
b5										0
b6			166.34							
b7				236.038						
b8							162.386			
b9		178.988								
b11						112.672				
b13					88.247					
b14								54.012		
b15						149.922				

2.5. Effect of Pethidine extent of bioavailability

The AUC had been calculated through Kinetica version 5 software, the results are shown in table 3.2.5.. The relationship of the AUC and the different baseline characters was statistically studied through the analysis of variance. The results are shown in the table 3.2.6.

Table 3.2.5: AUC of Pethidine for every subject

Subject	**T**	**C**	**AUC**	**AUCcum**	**AUMC**	**AUMCcum**	**Cmax**
	time interval between the dose and last sample (min)	ng/mL	ng/mL*min	ng/mL*min	ng/mL*(min)²	ng/mL*(min)²	(ng/ml)
1	120	233.445	441426	1.14E+06	2.76E+09	6.51E+09	257.028
2	120	116.429	854326	1.16E+06	3.24E+09	3.56E+09	199.988
3	185	126.511	555419	1.13E+06	4.96E+09	9.06E+09	158.319
5	213	304.299	1.43E+06	2.18E+06	1.50E+10	2.04E+10	304.299
6	120	257.538	673011	913416	4.06E+09	4.96E+09	257.538
7	120	88.341	268646	517622	1.53E+09	2.65E+09	110.656
8	180	99.896	539438	539438	5.83E+09	5.83E+09	99.896
9	120	215.113	608809	1.24E+06	3.53E+09	6.47E+09	268.069
11	136	217.572	887694	887694	7.24E+09	7.24E+09	217.572
13	125	32.68	48573	85239	3.34E+08	5.32E+08	355.867
14	160	109.15	523920	523920	5.03E+09	5.03E+09	109.15
15	120	196.038	416053	1.13E+06	2.56E+09	6.45E+09	266.243

Table3.2.6: Analysis of variance where the dependent variable is log AUC.

source	Sum-of - squares	DF	Mean-square	F-ratio	p-value
Mother age	0.272	1	0.272	0.869	0.382
Mother weight	0.709	1	0.709	2.268	0.176
Baby sex	0.524	1	0.524	1.677	0.236
baby weight	0.442	1	0.442	1.414	0.273
Apgar	2.742	1	2.742	8.773	0.021

The statistical results showed that neither the mother age or weight nor the baby sex or weight affect the area under curve ($p>0.05$). The only factor that was affected by the AUC is the Apgar score. The maternal AUC is the result of the drug concentration in function of time. Consequently, as the concentration increases, the AUC increases, and if the clearance time of Pethidine increases, the AUC increases simultaneously. As all the mothers have received the same dose, and as Pethidine is strongly correlated to lowering the Apgar score (Volikas I, Male D., 2001), consequently, the slower the mother metabolism results in higher transplacental passage resulting in low Apgar score.

2.6. Effect of Pethidine of high concentration (C max)

The relationship of Cmax with the different baseline characters of the mothers and newborn was also studied statistically through analysis of variance. The results are shown in table 3.2.7.

Table3.2.7: Analysis of variance where the dependent variable is Cmax

source	Sum-of - squares	DF	Mean-square	F-ratio	P-value
Mother age	0.079	1	0.079	0.846	0.388
Mother weight	0.082	1	0.082	0.878	0.38
Baby sex	0.241	1	0.241	2.585	0.152
baby weight	0.176	1	0.176	1.891	0.212
Apgar	0.751	1	0.751	8.052	0.025

The baseline characters including the mother age, mother weight, baby sex and baby weight do not affect the value of the maximum concentration reached in the mother. In consequence, the pharmacokinetics of Pethidine in mother during labor of pethidine can be comparable to the non pregnant women. In fact, the Pethidine half

life in women in labor is not different from that in non pregnant women, but the volume of distribution in the former is larger and the clearance is decreased (Morselli PL, Rovei V., 1982)**.**

As the concentration in mother increases, the Apgar score is lowered. This also can be explained that as the maternal Pethidine blood concentration increases, the trans-placental Pethidine passage is raised. This elucidates the rapid lowering of the fetal heart rate in case "4" where the maternal concentration was 42,017 ng/ ml. It should also be noted that the newborn metabolizes Pethidine seven times less than the adult on account of N-demethylation and N-oxidation pathways impairments (Caldwell J, Notarianni LJ., 1978).

The placental transfer of Pethidine can be reflected by the ratio of umbilical cord pethidine concentration to maternal Pethidine blood concentration sampled at the same time. This could not be performed in this study since in 4 cases of the study, post delivery Pethidine blood sampling from the mother was delayed.

2.7. Effect of the dose-delivery duration

The dose delivery duration is defined as the time period from Pethidine administration to the blood sampling from the umbilical cord.Using t-test, it was found that Apgar score is affected by Pethidine concentration in the newborn and dose duration (p-value <0.01) (table3.2.8).

Table 3.2.8: t-test for the evaluation of newborn concentration and dose duration on Apgar score

Variable	Coefficient	Std error	Std coeff	Tolerance	T	p
newborn conc	0.023	0.006	0.499	0.606	3.98	0.003
dose duration	0.037	0.008	0.555	0.606	4.427	0.001

2.8. Correlation Analysis

Using Pearson correlation matrix, the correlations between the factors Apgar score, baby concentration, dose duration, log AUC, log C (baby concentration) were evaluated (table 3.2.9).

Table 3.2.9: Correlation matrix between Apgar score, newborn concentration, dose duration, log AUC, log C

	Apgar	Baby conc.	Dose duration	Log AUC	Log C
Apgar	1.000				
Newborn conc.	-0.074	1.000			
Dose duration	-0.240	-0.542	1.000		
Log AUC	-0.293	0.081	0.194	1.000	
Log Cmax	-0.057	0.182	-0.021	0.939	1.000

The Pethidine concentration in the newborn is fairly negatively correlated to the dose duration, so as the time elapsed from IV injection to the sampling increases, the baby concentration decreases. In case “5”, the delivery took place 213 minutes after Pethidine administration, and pethidine concentration in the newborn was null. IV administration of Pethidine and the associated dose-delivery duration have not been previously addressed in published literature. There are published reports which address the IM administration of Pethidine. In one study, the maximum Pethidine

trapping in the baby was after 3 hours after 100mg IM Pethidine injection (Savona-Ventura C, Sammut M, Sammut C., 1991).Moreover, after a same dose, the ratio of fetal concentration: maternal concentration increases in the first 5 hours (Tomson G. et al., 1982).

2.9. Comparison between Pethidine and other labor analgesics

It was found that Pethidine has considerably sedative effect comparing to its analgesic effect (Matheson I, Nylander G. 1999). In fact, Pethidine results in less visual analogue scores of satisfaction for analgesia weighed against remifentanil in patient controlled analgesia (Blair JM, *et al*., 2005). And regarding the Apgar score at 1 to 5 minutes after delivery, Pethidine has demonstrated lower scores than remifentanil (Volikas I, Male D., 2001). However, other studies comparing the Apgar score in women taking remifentanil and women taking Pethidine have shown no significant difference between the 2 groups (Thurlow JA, *et al*., 2002). Moreover, remifentanil provides better analgesia than Pethidine or fentanyl only for the first hour after initiation of the treatment (Douma MR, *et al*. 2010).

On the other hand, epidural analgesia during labor has shown a significant superiority in terms of painrelief and side effects(Philipsen T, Jensen NH, 1990). Nevertheless, epidural bupivacaine does not extend the labor process nor increase the risk of instrumental delivery compared to parentral Pethidine (Philipsen T, Jensen NH, 1989).

3. Conclusion

A simple and rapid method of analysis for Pethidine in plasma has been validated using Liquid Chromatography tandem mass spectrometry. The retention time of Pethidine was 0.46 minutes.

It was found that the metabolism of Pethidine in mother is individual, and it is proposed to investigate more on the genetical mapping in order to decrease the incidence of fetal distress due to Pethidine. A negative correlation between the Pethidine concentration in the newborn and the dose-delivery duration was found, possibly reflecting differences in the pharmacokinetics of IV versus IM administration. It was also found that as the mother Pethidine concentration increases, the Apgar score will be lowered.

Pethidine IV is not safe for labor analgesia as 3 subjects out of 15 (20%) were withdrawn for fetal distress.

Consequently, the new recommendation for future study is that the number of cases will be added up, and in which the sampling will be more controlled and covers a wider interval of time, and perform a genetical mapping regarding Pethidine metabolism.