DETERMINATION OF FENTANYL IN HUMAN PLASMA WITH LIQUID PHASE EXTRACTION FOLLOWED BY HPLC METHOD AND ITS APPLICATION TO ANESTHESIA IN CORONARY ARTERY BYPASS GRAFT STUDY

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ABSTRACT

Although fentanyl has been widely used in cardiac anesthesia, there is limited data on the pharmacokinetic model for assessing the effects of a cardio-pulmonary bypass graft (CPBG). The aim of this study was to determine the fentanyl concentration in human plasma by using the HPLC-UV method. The investigation of population pharmacokinetics of fentanyl in CPB patients developed a simple model for predicting a fentanyl concentration-time profile which correlated with factors during the cardiac surgery.

The developed population pharmacokinetic modeling was applied to determine the optimal dosage regimen for patients during cardio-pulmonary bypass graft (CABG) surgery. The one-compartmental analysis of the data using the WinNonlin® program provided the best fit to find initial values of volume of distribution and clearance. The correlation between age, body weight, and dosage regimens was described by the Monolix® program. The primary result of the volume of distribution of fentanyl was correlated with the log of body weight in all groups. The population pharmacokinetics of a high dose regimen for volume of distribution and clearance were V_d=47.7 L and Cl=0.109 ml/min, respectively. The pharmacokinetic parameters of fentanyl from this study can be used as basic information for further study in related fields. However, the results were insufficient to explain the pharmacodynamic results of fentanyl, implying the importance of a titrating analgesic to the clinical effect. Thus, a more complete pharmacokinetic parameters model and suitable dosage regimen need more Thai patients' pharmacokinetic/pharmacodynamic data and the method of analysis for lower concentration measurements could be used.

KEY WORDS: FENTANYL / HPLC-UV / CORONARY ARTERY BYPASS GRAFT STUDY / POPULATION PHARMACOKINETICS

143 pages

การวัดระดับยาเฟนทานิลในเลือดมนุษย์ด้วยการสกัดด้วยของเหลวตามด้วย HPLC และการประยุกต์ใช้ในการศึกษาการสลบในผู้ป่วยผ่าตัดบายพาสเส้นเลือดหัวใจ

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บทคัดย่อ

ถึงแม้ว่ายาเฟนทานิลจะมีการใช้อย่างกว้างขวางในการผ่าตัดหัวใจ แต่ยังมีข้อมูลทางด้านเภสัช-จลนศาสตร์ที่จำกัดในคนไข้ที่ผ่าตัดบายพาสเส้นเลือดหัวใจ และการทำนายผลของขนาดยาที่มีต่อคนไข้อย่างถูกต้อง วัตถุประสงค์ของการศึกษานี้ คือ การหาปริมาณของยาเฟนทานิลในพลาสมาของคนไข้ด้วยวิธีวิเคราะห์ HPLC-UV และทำนายผลของระดับยาเฟนทานิลต่อเภสัชจลนศาสตร์ในประชากรจำนวนมาก โดยการใช้แบบจำลองอย่างง่ายในการทำนายความเข้มข้นของยาเฟนทานิลในขณะทำการผ่าตัดบายพาสเส้นเลือดหัวใจ และทำนายผลความสัมพันธ์ของเภสัชจลนศาสตร์ของยาต่อปัจจัยต่างๆ ในขณะทำการผ่าตัด ในระดับประชากร

ต้นแบบของเภสัชจลนศาสตร์ในประชากรและขนาดการให้ยาที่ดีที่สุด จะสามารถทำนายผลได้จากการหาขนาดยาเฟนทานิลที่ใช้ในคนไข้ผ่าตัดบายพาสเส้นเลือดหัวใจ โดยใช้รูปแบบ non-compartment with sparse model จากโปรแกรม WinNonlin® ได้นำมาใช้ในการหาค่าเริ่มต้นของปริมาตรการกระจายยาในร่างกาย กับ ค่าการกำจัดยาออกจากร่างกาย ตามด้วยโปรแกรม Monolix® ที่ใช้ในการหาความสัมพันธ์ของค่าพารามิเตอร์ทางเภสัชจลนศาสตร์กับ อายุ, น้ำหนักตัว และขนาดยาต่างๆที่ให้กับผู้ป่วย ผลจากการศึกษานี้พบว่า น้ำหนักตัวของผู้ป่วยมีความสัมพันธ์กับเภสัชจลนศาสตร์ของระดับยาเฟนทานิล ข้อมูลเภสัชจลนศาสตร์ของประชากรที่ได้รับยาในขนาดสูง พบว่า มีปริมาตรการกระจายตัวของยา เท่ากับ 47.7 ลิตร และ ค่าการกำจัดยาออกจากร่างกาย เท่ากับ 0.109 มล.ต่อนาที ตามลำดับ ซึ่งตัวแปรทางเภสัชจลนศาสตร์ของยาเฟนทานิลจากการศึกษานี้ สามารถใช้เป็นพื้นฐานสำหรับการศึกษาอื่นที่เกี่ยวข้อง อย่างไรก็ตามผลการทดลองนี้ยังไม่สมบูรณ์ในส่วนของผลด้านเภสัชพลศาสตร์ ซึ่งมีความสำคัญในการทำนายผลทางคลินิก ดังนั้นสมการตัวแปรทางเภสัชจลนศาสตร์ที่สมบูรณ์กับขนาดยาที่เหมาะสม จึงต้องการข้อมูลเภสัชจลนศาสตร์และเภสัชพลศาสตร์จากผู้ป่วยไทยที่มากกว่านี้ และต้องการวิธีวิเคราะห์ที่สามารถวัดความเข้มข้นของเฟนทานิลในระดับที่ต่ำกว่านี้ จึงจะทำนายผลความสัมพันธ์ของเภสัชจลนศาสตร์/เภสัชพลศาสตร์ของยาเฟนทานิลในร่างกายผู้ป่วยที่กำลังผ่าตัดบายพาสเส้นเลือดหัวใจได้

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LIST OF ABBREVIATIONS

µg	Microgram(s)
µL	Microliter(s)
ADME	Absorption, Distribution, Metabolism, Excretion
ALC	Alkaline criteria
ASA	American Society of Anesthesiologist
AUC	Area under the curve
BIC	Swartz criteria
BQL	bBelow quantitative limit
℃	Degree celsius
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
Cl	Clearance
CNS	Central nervous system
conc.	Concentration
CPB	Cardio-pulmonary bypass
CROs	Contract research organization
cm	Centimeter(s)
CTS	Clinical trial simulation
CV	Coefficient of variation
CYP	Cytochrome P
et al.	Et alii, and others
FDA	Food and Drug Administration
GAM	Generalized additive modeling
GC-MS	Gas chromatography-mass spectrophotometry
HDL	High density lipoprotien
HPLC	High performance liquid chromatography

LIST OF ABBREVIATIONS (cont.)

HPLC-UV	High performance liquid chromatography-UV detector
IHD	Ischemic heart disease
hr	Hour
i.d., ID	Internal diameter
IV	Intravenous
IS	Internal standard
Kg	Kilogram
L	Litter
LC-MS	Liquid chromatography-mass spectrophotometry
LDL	Low density lipoprotien
LL	Log-likelihood
LLE	Liquid-liquid extraction
LLOQ	Lower limit of qauntification
mg/L	Milligram per litter
mg/kg/day	Milligram per kilogram per day
ml/min	Milliliter per minute
NDAs	New drug applications
ng	Nanogram(s)
PD	Pharmacodynamic
PK	Pharmacokinetic
PK/PD	Pharmacokinetic/pharmacodynamic
PTCA	Percutaneous transluminal coronary angioplasty
QC	Quality control
R	Coefficient of correlation
R^2	Coefficient of determination
R.E.	Relative error
RPC	Reverse phase chromatography

LIST OF ABBREVIATIONS (cont.)

RP-HPLC	Reverse phase-high performance liquid chromatography
rpm	Round per minute
R.S.D	Relative standard error
S.D.	Standard deviation
SOP	Standard operating system
$t_{1/2\beta}$	Elimination half-time
TG	Triglyceride
UHPLC	Ultra high performance liquid chromatography
ULOQ	Upper limit of quantification
USP	United State Pharmacopiea
UV	Ultraviolet
UV-VIS	Ultraviolet-visible
V_d	Volume of distribution

CHAPTER I
INTRODUCTION

Atherosclerosis is the underlying pathologic evidence of Coronary Artery Disease (CAD). The causes of the disease are not yet exactly known, but certain risk factors are linked with the development of atherosclerotic plaques (1). The effective treatments for CAD patients can be categorized as fellows: 1) medication such as thrombolytic agent, 2) procedure such as Percutaneous Transluminal Coronary Angioplasty (PTCA), Coronary stent implantation, Coronary Artery Bypass Graft (CABG) surgery, and 3) combination of medications and procedures. The choice of treatment modalities are based primarily on the severity of the disease (2).

Fentanyl, an opiate analgesic is required during surgery or to support patients undergoing mechanical ventilation in the intensive care unit. The combination with benzodiazepines has proven to be successful when both sedation and analgesia are needed. Plasma concentration measurements of the drugs can help to improve the clinical diagnosis. However, a determination of fentanyl in biological fluids was difficult because only poor peak separation from the benzodiazepine (such as midazolam) was obtained. Thus, quantification methods of drug in pharmacokinetic studies need to be sensitive and specific.

There are literature reports describing the quantification of fentanyl in human plasma using liquid-liquid extraction (LLE) and using HPLC-UV with a cyano-HPLC column (3-4). However, the cyano-HPLC column was not tolerated at high concentration of buffer solution with low pH (< 3). Moreover, the extracted samples showed poor absorbance by UV-VIS region. The analytical methods based on mass spectrometric detection (GC-MS, LC-MSMS) can be found in the literatures (5-6). The methods were more specific and sensitive than HPLC, but the cost of analysis was more expensive. It is necessary to establish a suitable method for measuring the low concentration of fentanyl in human biological fluids. In the present study, a simple, sensitive and rapid reverse phase HPLC method was developed and validated

to quantify fentanyl in human plasma. This method was applied to a pharmacokinetic study in human.

The population pharmacokinetic about fentanyl was described in neonate and burn patient (7-9) but was not yet been described in adult CABG patients. There is also limited data on the dosage regimen for CABG patients. This study was designed to compare the pharmacokinetics in different dosing regimens in 48 bypass patients. The plasma concentration time profile of each patient was fitted to the individual dosage regimen. The population pharmacokinetic parameters were investigated using WinNonlin® and Monolix® program.

WinNonlin® is the industry-standard analysis software for pharmacokinetic, pharmacodynamic, and non-compartmental analysis. In addition to its extensive library of built-in PK, PD and PK/PD models, WinNonlin® supports custom, user-defined models to address any kind of data. Monolix® is the free software dedicated to the analysis of nonlinear mixed effects models. In this research WinNonlin® software was used to determination of initial values such as volume of distribution and clearance in each pair of groups. Then, Monolix® software was used to evaluation of the correlation between fentanyl pharmacokinetics and factors of interest such as age, sex, body weight, the difference of dosage regimens.

The specific aims of this study are 1) To develop and validate a HPLC assay for the quantification of fentanyl in human plasma and 2) To demonstrate the population pharmacokinetics in cardio-pulmonary bypass graft patients.

CHAPTER II
LITERATURE REVIEW

This research describes the determination of fentanyl in human plasma with liquid phase extraction followed by HPLC method and its application in pharmacokinetic study of coronary artery bypass graft patients. The related literatures for this study include:

1. Coronary Artery Disease and Coronary Bypass Graft.
2. Pharmacokinetics / Pharmacodynamics of fentanyl IV injection.
3. HPLC method development followed by FDA guidances.
4. Population pharmacokinetics in research by population PK program.

2.1 Coronary Artery Disease and Coronary Bypass Graft

Coronary artery disease (CAD) or Ischemic heart disease (IHD) or Myocardial infarction (MI) results from an imbalance between the flow of blood to myocardium (supply) and the metabolic need of the myocardium (demand). Supply ischemic results from functional or structural abnormalities in the coronary arteries, reducing blood flow in the region perfuse by the vessels. Demand ischemic results from increased heart rate, contractility. Myocardial ischemic is by definition, reversible and presents clinically as angina pectoris. Severe and prolong myocardial ischemic result in irreversible injury or infraction of tissue (10).

2.1.1 Risk factor

The term of risk factor was reported by the Framingham study in the 1960's. The spectrum of cardiovascular risk factor can be divided into two major categories: that are modifiable such as smoking, cholesterol level, and evaluated blood pressure; and those cannot be modifiable such as family history, gender, age.

Serum cholesterol is the most common and highly manifestation of atherosclerosis. Hyperlipidemia is an abnormal level of blood lipid and lipoprotein. Plasma level of total cholesterol, low density lipoprotein (LDL) cholesterol and triglyceride are considered positive associate with the incidence of the CAD. High level of LDL promotes atherosclerosis because LDL deposits cholesterol on the artery walls. Whenever there is a higher blood cholesterol level, high density lipoprotein (HDL) helps to clear cholesterol from the arteries to the liver for excretion. Triglyceride (TG), compound of fatty acid, is bound to glycerol, stored as fat by the body, and carried on very low density lipoprotein (LDL) molecules (11).

Diabetes mellitus contributes to CAD in several ways. The disease is associated with a greater incidence of high blood lipids, high blood pressure and obesity. The disease affects both small and large blood vessels. Hyperglycemia altered platelet function, and elevated fibrinogen is also affected platelet function (12).

Hypertension is one of the most prevalent and powerful factors that contribute to atherosclerotic cardiovascular disease. The disease is classified as a systolic blood pressure greater than 140 mmHg or diastolic blood pressure greater than 90 mmHg. Hypertensive affects all aspects of cardiovascular function.

Cigarette smoking is a primary cause of CAD. The carbon monoxide produced may damage the blood vessel lining, promoting cholesterol deposits. Nicotine is also a stimulant for constriction of the coronary vessels, thus reducing blood flow to the heart. Additionally, sympathetic stimulation causes catecholamine release, increasing work and oxygen demand of the heart by increasing blood pressure and heart rate.

Physical inactivity or lack of exercise is related to high risk of CAD. Cardiovascular benefits of exercise increases availability of oxygen to the heart muscle, decrease oxygen demand and cardiac workload and increase myocardial function and electrical stability. Family history is generally associated with 1.5 to 2 fold increase in the risk factor of CAD among first degree relative (13).

2.1.2 Pathophisiology (Figure 2.1)

The coronary circulation supplies to the heart by left and right coronary arteries, which originate via their ostia from the left and right aortic sinuses of Valvasa

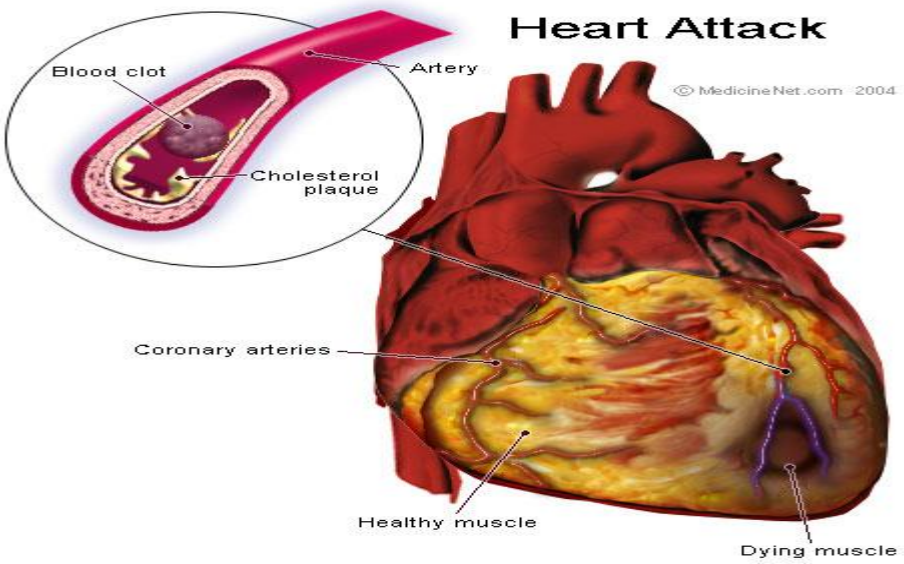


Figure 2.1 Pathophysiology of coronary atherosclerosis.

, locate just distal to the aortic valve. The short left coronary artery divides into left anterior descending and left circumflex branches. The anterior portion or the left ventricle and the inter-ventricular septum are supplied by the left anterior descending coronary artery and its left diagonal and septum branches. The left circumflex coronary artery and its circumflex marginal branch supply the lateral left ventricle. The right coronary artery supplied the right ventricle (14).

The coronary artery wall is composed of three layers. The intima, a single layer of endothelial cells that lines the lumen of the artery, the media, a dense array of smooth muscle cells surrounded by loosely arranged connective tissue, and the connective tissue layer, made up of smooth muscle, collagen and mucopolysaccharide (15).

The major cause of CAD is coronary atherosclerosis, a process that develops as a response of the vessel wall to chronic multifactorial injury and leads to the formation of atherosclerosis plaques. A typical coronary artery plaque is composed of various mixtures of fiber cap and lipid. Initially, atherosclerosis is a focal disease. There is a predilection for formation of atherosclerotic plaques adjacent to branch points in areas of low velocity flow and low shear stress. It is postulated that the flow patterns in such regions promote endothelial dysfunction and increased contact of endothelium with monocytes and platelets. The areas of predilection for severe atherosclerosis in the coronary system include the proximal and distal right coronary arteries. Established atherosclerosis involves three layers of the arterial wall such as intimal thickening disease, medial degeneration and weakening and intima fibrosis, with lymphocytic inflammatory infiltrates.

Atherosclerosis disease leads to extensive remodeling of the vessel wall. Dilatation of the vessel occurs, in such a way that the lumen is maintained despite the presence of intima plaque, which may develop in an eccentric or concentric pattern. Luminal narrowing occurs only when atherosclerosis disease is advanced. The area of severe narrowing often develops in the setting of multifocal disease. There is approximately 50 percent narrowing of luminal diameter that effects coronary blood flow.

The pathology of CAD may be found as a single vessel, double vessels, triple vessels, or multiple lesions in one vessel. The proximal left anterior descending

coronary artery is the most common site affected. The second is the proximal right coronary artery, followed in diminishing frequency by the left circumflex artery and the distal right coronary artery. The left main artery is involved less often but a significant stenosis in this segment of the coronary artery is the greatest danger to the patients (16).

Myocardial oxygen demand is increased by exercise, emotional stress, and eating heavy meal. Coronary atherosclerosis or vasospasm may prevent adequate coronary vasodilatation which results in myocardial ischemia. Ischemia is reversible, but if myocardial blood flow is not increased or oxygen demands are not reduced, ischemia can lead to cell death (17).

Chest pain or angina pectoris is the common presented symptom. However, many asymptomatic patients present for an evaluation with coronary factors. The initial presentation of pain is considered to be the most significant prognostic factor for CAD. Classical angina pectoris reflects an imbalance in myocardial oxygen supply and demand (18).

2.1.3 Treatment modalities of CAD.

There are several methods to treat patients with CAD such as medication, various procedures such as percutaneous transluminal coronary angioplasty (PTCA), coronary stent implantation, coronary artery bypass graft (CABG) surgery. The effectiveness of these methods was depended on many factors, for example: severity, myocardial capacity, and duration of seeking heath care providers and also patients' self care practice.

Coronary bypass graft surgery is a major invasive treatment for occlusive coronary artery disease. The first coronary artery bypass operation was done by Sabiston in 1962. A bypass to the right coronary artery was performed without the use of extracorporeal circulation. Hallman, et al. performed the first successful direct reconstruction of a congenital coronary artery abnormal by using a dacron tube graft in 1963. In 1965 Garrett performed the first successful CABG. While performing an endarterectomy, he encountered technical difficulties and was forced to bypass the left anterior descending artery (Figure 2.2).

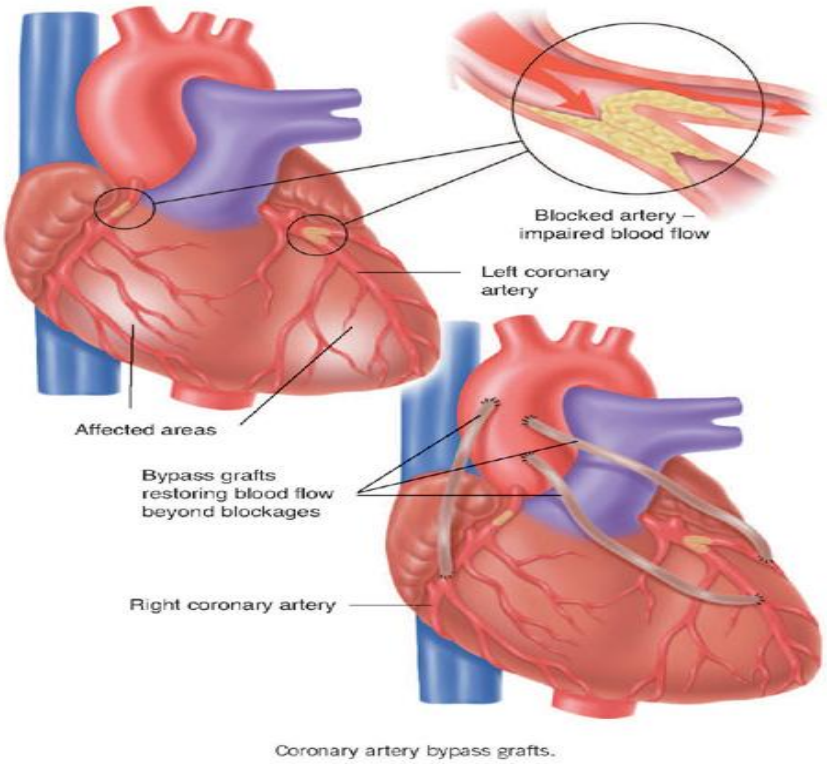


Figure 2.2 The picture of Cardio-pulmonary bypass grafts (CABG).

The major goal of CABG is relief of myocardial ischemic and relief of disabling angina pectoris. CABG is a concomitant procedure for patients with cardiac failure resulting from ventricular aneurysm, ventricular septal perforation, papillary muscle rupture disease.

Although CABG is now commonly performed, the procedure, like other open heart operation, is not without risks. Calcification, small vessel, and diffuse coronary atherosclerosis add risk of procedure. Diabetes, hypertension, collagen disease, long-term steroid use and female gender are additional risk factors as is the degree of myocardial dysfunction, should be assessed preoperatively (19).

1.1.4 Anesthetic management during cardio-pulmonary bypass (CPB) (20)

The goals of anesthetic management during cardiopulmonary bypass (CPB) are diverse. (Figure 2.3) Anesthetic agents and manipulations affect multiple areas of the patient's physiology. These include metabolic rate and oxygen consumption, electrical activity in the brain, cerebral blood flow, pulmonary physiology, systemic vascular resistance and arterial perfusion pressure, and the hormonal and metabolic stress responses to CPB. Because of these widespread effects, anesthetic techniques may have important effects on the response of multiple organ systems to CPB, and thus on patient outcome.

On the most elementary level, anesthetic management during bypass must ensure that the patient is anesthetic deeply enough to prevent recall and paralyzed to prevent movement. Circulating and tissue levels of anesthetics and ancillary drugs must be adequate to ensure this. However, anesthetic agents are given for multiple purposes during bypass other than rendering the patient unconscious and immobile. Anesthetics are also given to decrease systemic vascular resistance, minimize pulmonary stress response, attenuate hormonal and metabolic responses to CPB, and protect the brain. Depending on institutional practices and the individual involved, anesthetic management may also involve managing acid base balance during bypass, controlling arterial blood pressure, managing haematocrit levels and weaning from bypass. These functions will obviously involve close cooperation with the surgeon and the anesthesiologist.

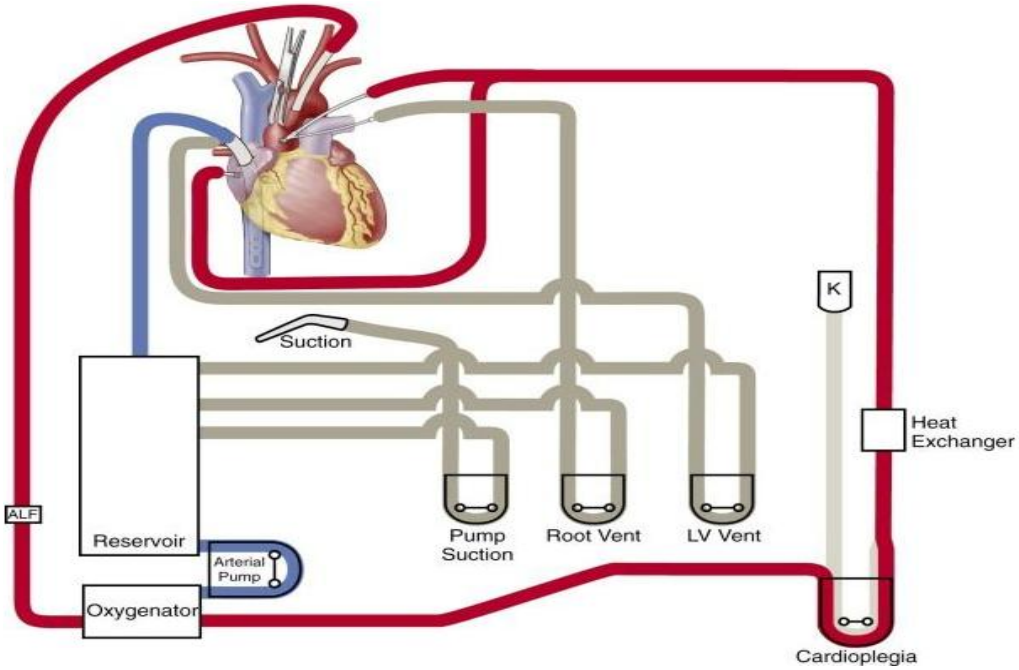


Figure 2.3 Cardiopulmonary bypass circuit.

Anesthesiologists are responsible for these cases must be knowledgeable about bypass and CPBG so that they can make useful contributions to overall management of these patients. Because of the impact of anesthetic agents on critical organ systems during CPB, it is particularly important that anesthesiologists understand the complex physiology of bypass, particularly the unique problems of CPB in adults. It is also particularly important that the surgeon communicates information about the progress of the procedure and concerns that develop during bypass to the anesthesiologist in order to facilitate cooperative patient care efforts.

2.1.5 Influence of cardiopulmonary bypass on anesthetic agents (20)

The levels of anesthetic agent in the blood, their protein binding, their metabolism, and their distribution all may be altered by CPB because of the extensive heamodilution, profound levels of hypothermia and changes in organ blood flow that occur with bypass. Such changes in the levels of anesthetic agents will of course later their effects. Unfortunately, the influence of CPB on tissue and circulating levels of anesthetic agents has been studies for only a few anesthetic agents and such studies have not been carried out in population pharmacokinetics.

There are many types of anesthetic agents such as inhalation agents, intravenous agents. In this research, we will focus on intravenous agent especially fentanyl and benzodiazepines. The serum levels of the various intravenous anesthetic agents and ancillary anesthetic agents can change radically during CPB. For example, plasma levels of commonly used anesthetic agents such as fentanyl have been demonstrated to decrease dramatically when cardiopulmonary bypass is begun in adults (21). Although fentanyl has been widely used in cardiac anesthesia, no complete pharmacokinetic model that has assessed the effect of CPB and that has adequate predictive accuracy has been defined. The aims of these investigations were determine whether CPB had clinically significant impact on fentanyl pharmacokinetics and predict fentanyl concentrations during cardiac surgery using CPB. The effect of CPB on fentanyl kinetics is clinically insignificant by using three-compartment model (22).

With such drugs, pH changes during bypass may considerably alter protein binding and thus free drug fraction in the serum. Depending on the amount of

anesthetic drugs bound to tissue, especially brain tissue, such changes may or may not significantly alter anesthetic effects during bypass.

Drug level of hypnotic anesthetic agents such as benzodiazepines and barbiturates are affected differently during CPB depending on their protein binding characteristics. Benzodiazepines such as midazolam and lorazepam show arupt decrease in plasma levels with the onset of bypass followed by increase in levels during the warming period (23). However, this research not to mention about benzodiazepine pharmacokinetics.

For pharmacodynamics; Changes in drug effect occurring as a result of CPB are only beginning to be explored. The ability of drug to produce its effect is contingent on the ability of free drug to reach its receptor and activate the receptor to transducer the signal. A number of factors any affect the ability of the drug to reach and interact with its receptor during cardiac surgery and CPB.

2.2 Pharmacokinetics / Pharmacodynamics of fentanyl IV injection.

2.2.1 Basic principal of definition of terms (24)

To understand how CPB may alter drug effects, it is nessesary to have an understanding of the basic principles of pharmacokinetics and pharmacodynamics.

2.2.1.1 Pharmacokinetics

Pharmacokinetics may be defined as the mathematical description of the processes by which a drug is handle once introduced into the body (i.e., what the body does to the drug). Because most drugs given during the conduct of cardiac surgery are given intravenously, this discussion focuses on a description of pharmacokinetics terms after intravenous administration.

Pharmacokinetics is divided into several areas which includes the extent and rate of Absorption, Distribution, Metabolism and Excretion. This is commonly referred to as the ADME scheme.

- *Absorption* is the process of a substance entering the blood circulation.

- *Distribution* is the dispersion or dissemination of substances throughout the fluids and tissues of the body.
- *Metabolism* is the irreversible transformation of parent compounds into daughter metabolites.
- *Excretion* is the elimination of the substances from the body. In rare cases, some drugs irreversibly accumulate in a tissue in the body.

Pharmacokinetics describes how the body affects a specific drug after administration. Pharmacokinetic properties of drugs may be affected by elements such as the site of administration and the concentration in which the drug is administered. These may affect the absorption rate (25).

After injection of a single intravenous dose of drug (i.e., induction of the anesthetic state at the beginning of cardiac surgery), a number of processes are immediately initiated that reduce drug levels. Drug is delivered to and taken up by tissue within the body – a process known as *distribution*. Distribution occurs first to tissues that are highly perfused, such as the brain, heart, lungs, liver, and kidneys, where uptake by the tissue occurs. Tissue uptake is variable depending on protein binding and the lipid solubility of the drug. Thereafter, further uptake of drug occurs into tissues that are less well perfused, such as muscle and fat. Simultaneously, drug is delivered to organs where *elimination* by biotransformation and excretion can occur. For most drugs used during cardiac surgery, this elimination occurs at a constant fraction of drug remaining in the body per unit of time, so-called first-order kinetics.

Characterization of concentration versus time profiles (Figure 2.4) for intravenously administered drugs also allows the derivation of other pharmacokinetic parameters such as the volume of distribution, clearance, and elimination haft-time.

Volume of distribution

Volume of distribution (V_d) may be defined as that volume of fluid into which a drug would be administered to produce the observed concentration of drug in plasma. It does not correspond directly to any particular tissue compartment but rather is useful in predicting drug levels based on pharmacokinetic parameters. V_d is used to characterize the total volume of distribution, whereas V_c describes the volume

$$r = \frac{-d[A]}{dt} = -k[A]$$

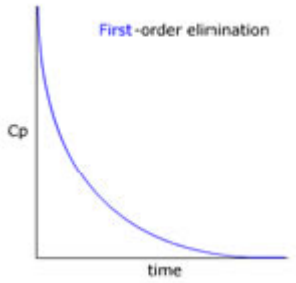


Figure 2.4 The first order equation and graph concentration versus time profile.

of the central compartment, or the initial volume of distribution (also term V_i). V_{ss} describes the volume of distribution when steady state plasma concentrations of drug are achieved.

Clearance

Clearance (*Cl*) refers to the removal of drug from the body, usually by way of the central of compartment, and is expressed as volume of blood cleared of drug per unit of time.

Elimination Half-Time

Elimination half-time ($t_{1/2\beta}$) is the time required for the concentration of drug in plasma to decline by one half. It can be determined by examining the elimination portion of the concentration versus time curve or by substituting the relevant parameters in the equation

$$(t_{1/2\beta}) = 0.693 * V_d / Cl$$

Similarly, distribution half-times can also be determined by examining the distribution phase of the concentration versus time curve.

Drugs with short elimination half-times are characterized by small volume of distribution and/or rapid clearance. Drugs with a long elimination half-time tend to be highly lipid soluble (most anesthetic agents) with a large volume of distribution and/or slow rate of elimination.

The degree to which drug effect is terminated depends on the rapidity of drug redistribution to the central compartment once the injection stops and the capacity of elimination processes to clear the drug. Although important after injection of a single dose, this concept is also of importance when drugs are given by continuous infusion or in repeated doses. If drug administration is greater than the body's ability to clear it, drug accumulation will occur, leading to prolonged drug effect.

Termination of drug effect is highly dependent on clearance mechanism. For most drugs this involves some degree of liver metabolism and/or renal excretion. Lipophillic drugs are metabolized in the liver in two stages: Phase I and Phase II reaction. Phase I reactions convert lipophillic drugs to more water soluble-compounds and Phase II reactions couple the drug (or its metabolites) to an

endogenous substrate such as sulphate, acetate, or glucuronide to form a highly polar water-soluble compound more easily excreated (26).

The ability of the liver metabolize a drug in the absence of limitations imposed by the hepatic blood flow or drug-protein binding is termed *intrinsic hepatic clearance*. The *hepatic extraction ratio* is the fraction of drug contained in hepatic arterial blood that is removed as it passes through the liver. For drugs with a high extraction ratio, a large percentage of the drug is removed per unit of time as it traverses the liver bed. These two concepts are related by the equation

$$Cl_{hepatic} = Q * \frac{Cl_i}{(Q + Cl_i)} = Q \frac{(C_a - C_v)}{C_a} = Q * E$$

where $Cl_{hepatic}$ is the hepatic clearance rate of drug, Q is the liver blood flow, Cl_i is the intrinsic hepatic clearance, C_a is the arterial drug concentration, C_v is the venous drug concentration, and E is the hepatic extraction ratio.

Metabolism of drugs with a high extraction ratio such as fentanyl may be critically affected by changes in liver blood flow (27).

Pharmacokinetic analysis is performed by noncompartmental (model independent) or compartmental methods. Noncompartmental methods estimate the exposure to a drug by estimating the area under the curve of a concentration-time graph. Compartmental methods estimate the concentration-time graph using kinetic models. Compartment-free methods are often more versatile in that they do not assume any specific compartmental model and produce accurate results also acceptable for bioequivalence studies (25).

Noncompartmental analysis

Noncompartmental PK analysis is highly dependent on estimation of total drug exposure. Total drug exposure is most often estimated by area under the curve (AUC) methods, with the trapezoidal rule (numerical differential equations) the most common area estimation method. Due to the dependence on the length of 'x' in the trapezoidal rule, the area estimation is highly dependent on the blood/plasma sampling schedule. That is, the closer your time points are, the closer the trapezoids are to the actual shape of the concentration-time curve.

Compartmental analysis (Figure 2.5)

Compartmental PK analysis uses kinetic models to describe and predict the concentration-time curve. PK compartmental models are often similar to kinetic models used in other scientific disciplines such as chemical kinetics and thermodynamics. The advantage of compartmental over some non-compartmental analyses is the ability to predict the concentration at any time. The disadvantage is the difficulty in developing and validating the proper model. Compartment-free modeling based on curve stripping does not suffer this limitation. The simplest PK compartmental model is the one-compartmental PK model with IV bolus administration and first-order elimination. The most complex PK models (called PBPK models) rely on the use of physiological information to ease development and validation.

1.2.1.2 Pharmacodynamics

Pharmacodynamics describes how a drug interacts with the body to produce its effects. Most drugs produce these effects by interaction with a specific *receptor* (i.e., that macromolecular component of the organism with which the chemical interacts (28). Activation of the receptor leads to change intracellularly, often through activation of secondary messengers, which leads to changes in cell function (e.g.,muscle contraction). The most numerous type of receptor is protein, although other type exist (e.g., nucleic acids). Among protein receptors, those that serve as endogenous regulatory ligands are particularly important because drugs that interact with these receptors will produce physiological effects similar to that which occurs in nature when an endogenous ligand stimulates the receptor (29-32). Such agents are referred to as *agonists*. Drugs that bind to the receptor but do not mimic but rather interfere with the binding of the endogenous ligand and process no intrinsic regulatory activity of their own are termed *antagonist*.

Whether a drug acts as an agonist or antagonist depends on its structure, and this is exploited in drug design. The degree to which a compound is capable of mimicking the effect of the endogenous ligand is also a function of the number and affinity of the receptor(s) for the drug and the concentration of drug.

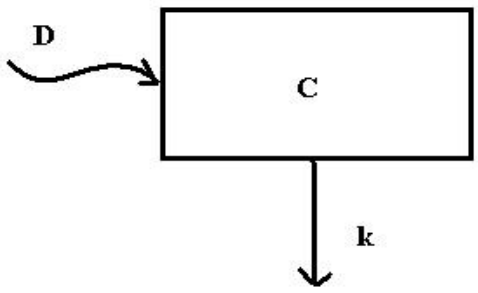


Figure 2.5 The one-compartment model (IV bolus).

2.2.2 Background information on fentanyl (33-35)

2.2.2.1 Structure and chemical properties

Fentanyl is a synthetic phenylpiperidine-derivative opiate agonist. (Figure 6) Fentanyl citrate is N-phenyl-N-(1-(2-phenylethyl)-4-piperidinyl) propanamide.citrate salt. The empirical formula of fentanyl citrate is $C_{22}H_{28}N_2O.C_6H_8O_7$ and its molecular weight is 528.59 (fentanyl molecular weight 336.47). Fentanyl citrate is a crystalline powder that is meilting point 149-151 °C, bitter taste. One gram of fentanyl citrate dissolves in about 40 ml of water, soluble in methanol, sparingly soluble in chloroform.

2.2.2.2 Pharmacology

Fentanyl citrate shares the actions of the opiate agonists. When given in equivalent analgesic doses, fentanyl is similar to morphine and meperidine in its respiratory effects except that respiration of healthy individuals returns to normal more quickly after fentanyl than after either of the other drugs. In controlled clinical trials employing an acute pain model in opiate-naïve (nontolerant) patients, a fentanyl transdermal system dosage of 100 mcg/hour provided analgesia approximately equivalent to that of 60 mg of IM morphine sulfate daily.

2.2.2.3 Pharmacodynamics and Pharmacokinetics

Pharmacodynamics

Fentanyl is analgesic effect as well as use general anesthetic. Although pharmacodynamiccally similar to meperidine and morphine, fentanyl exhibits little hypnotic activity, and histamine release rarely occurs. Fentanyl binds with stereospecific receptors at many site within CNS, increase pain threshold, alters pain reception, inhibits ascending pain pathways.

Pharmacokinetics

Bioavailability IV 100%

Following parenteral administration, the action of fentanyl is more prompt and less prolonged than that of morphine or meperidine. The onset of action following IV administration is rapid; peak analgesia occurs within several minutes and the duration of analgesic is 30-60 minutes after a single dose of up to 100 mcg. In nontolerant patients, minimum effective analgesic serum generally increase as serum concentrations range from 0.2-2 ng/ml. Serum concentrations of the drug may be

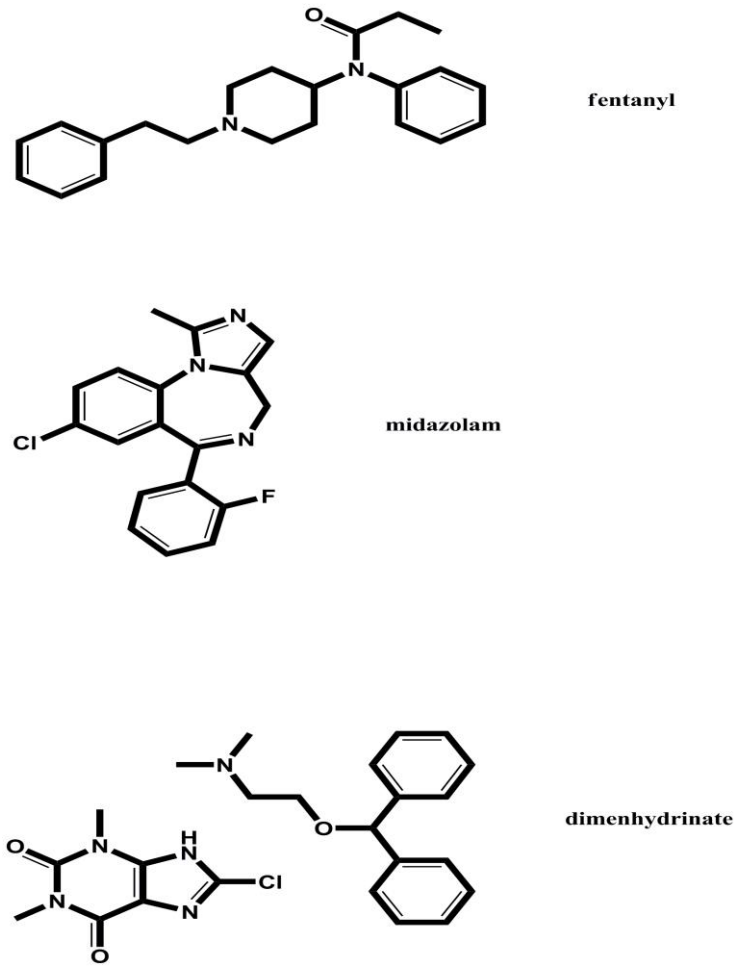


Figure 2.6 Chemical structures of fentanyl, midazolam and dimenhydrinate.

be useful clinically, but they should not be relied on sole indicators of efficacy or toxicity since they do not reflect individual patient sensitivity to fentanyl.

Distribution**:**

Following IV administration, fentanyl distributes rapidly from blood into the lungs and skeletal muscle and more slowly into deeper fat compartments. The drug then redistributes slowly from these tissues into systemic circulation. Large single doses or repeated doses can result in substantial accumulation of the drug, potentially resulting in an extended duration of effect. Fentanyl is 80-85% protein bound in plasma, principally to alpha1-acid glycoprotein but also to albumin and lipoproteins. The free fraction of fentanyl in plasma increases with acidosis. The mean volume of distribution of the drug at steady state has been reported to be 4 L/kg. Fentanyl is distributed into milk in plasma.

Metabolism**:**

Fentanyl citrate is metabolized extensively in the liver and the intestinal mucosa. Animal studies indicate that the drug undergoes oxidation via the microsomal enzymes in the liver and intestinal mucosa (principally cytochrome P-450(CYP) isoform 3A4) to form norfentanyl ;the drug also undergoes hydrolysis to form 4-N-anilinopiperidine and propionic acid. Norfentanyl has been shown to be pharmacologically inactive in animal studies. Fentanyl is excreted in urine as inactive metabolite and as unchanged drug.

Excretion**:**

Serum fentanyl concentrations decreased rapidly to about 20% of peak concentrations within 5 minutes after IV administration of the drug. Serum concentrations then decreased more slowly during the next 10-20 minutes and stabilized at low concentration by 2 hours. Concentrations then decreased very slowly and the drug could be detected for at least 6 hours after administration. Less than 10%of dose is excreted in urine unchanged and only about 1% is excreated in the feces as unchanged drug.

2.3 HPLC method development followed by FDA guidance. (36-43)

2.3.1 Method development

2.3.1.1 Method development background

A bioanalytical method is a set of all of the procedures involved in the collection, processing, storing, and analysis of a biological matrix for an analyze. Analytical methods employed for quantitative determination of drugs and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data that in turn are used in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetics. Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification.

Choice of extraction procedure, which is time economical, gives the highest possible recovery without interference at the elution time of the analyte of interest and has acceptable accuracy and precision. Method performance is determined primarily by the quality of the procedure itself. The two factors that are most important in determining the quality of the method are selective recovery and standardization.

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use. The fundamental parameters for this validation include accuracy, precision, selectivity, sensitivity, reproducibility and stability. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method.

2.3.1.2 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. The purity of the reference standard used to prepare spiked samples can affect study data. For this reason, an authenticated analytical reference standard of known identity and purity should be used to prepare solutions of known concentrations. If possible, the reference standard should be identical to the analyte. When this is not possible, an established chemical form (free base or acid, salt or ester) of known purity can be used. Three types of reference standards are usually used: 1) certified reference standards (e.g., USP compendial standards); 2) commercially supplied reference standards obtained from a reputable commercial source; and/or 3) other materials of documented purity custom-synthesized by an analytical laboratory or other noncommercial establishment. The source and lot number, expiration date, certificates of analyses when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference standard.

2.3.1.3 Principles of Bioanalytical Method Validation and Establishment

The fundamental parameters to ensure the acceptability of the performance of a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability.

A specific, detailed description of the bioanalytical method should be written. This can be in the form of a protocol, study plan, report, and/or SOP.

Each step in the method should be investigated to determine the extent to which environmental, matrix, material, or procedural variables can affect the estimation of analyte in the matrix from the time of collection of the material up to and including the time of analysis.

It may be important to consider the variability of the matrix due to the physiological nature of the sample. In the case of LC-MSMS-based procedures, appropriate steps should be taken to ensure the lack of matrix effects

throughout the application of the method, especially if the nature of the matrix changes from the matrix used during method validation.

A bioanalytical method should be validated for the intended use or application. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report).

Whenever possible, the same biological matrix as the matrix in the intended samples should be used for validation purposes. (For tissues of limited availability, such as bone marrow, physiologically appropriate proxy matrices can be substituted.)

The stability of the analyte (drug and/or metabolite) in the matrix during the collection process and the sample storage period should be assessed, preferably prior to sample analysis.

For compounds with potentially labile metabolites, the stability of analyte in matrix from dosed subjects (or species) should be confirmed.

The accuracy, precision, reproducibility, response function and selectivity of the method for endogenous substances, metabolites, and known degradation products should be established for the biological matrix. For selectivity, there should be evidence that the substance being quantified is the intended analyte.

The concentration range over which the analyte will be determined should be defined in the bioanalytical method, based on evaluation of actual standard samples over the range, including their statistical variation. This defines the standard curve.

A sufficient number of standards should be used to adequately define the relationship between concentration and response. The relationship between response and concentration should be demonstrated to be continuous and reproducible. The number of standards used should be a function of the dynamic range and nature of the concentration-response relationship. In many cases, six to eight concentrations (excluding blank values) can define the standard curve. More standard concentrations may be recommended for nonlinear than for linear relationships.

The ability to dilute samples originally above the upper limit of the standard curve should be demonstrated by accuracy and precision parameters in the validation.

In consideration of high throughput analyses, including but not limited to multiplexing, multicolumn and parallel systems, sufficient QC samples should be used to ensure control of the assay. The number of QC samples to ensure proper control of the assay should be determined based on the run size. The placement of QC samples should be judiciously considered in the run.

For a bioanalytical method to be considered valid, specific acceptance criteria should be set in advance and achieved for accuracy and precision for the validation of QC samples over the range of the standards.

2.3.1.4 Specific Recommendations for Method Validation

The matrix-based standard curve should consist of a minimum of six standard points, excluding blanks, using single or replicate samples. The standard curve should cover the entire range of expected concentrations.

Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit.

LLOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The LLOQ should be established using at least five samples independent of standards and determining the coefficient of variation and/or appropriate confidence interval. The LLOQ should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection and/or the low QC sample. The highest standard will define the upper limit of quantification (ULOQ) of an analytical method.

For validation of the bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within ±15% of the theoretical value, except at LLOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ, where it should not exceed 20% of the CV. Other methods of assessing accuracy and precision that meet these limits may be equally acceptable.

The accuracy and precision with which known concentrations of analyte in biological matrix can be determined should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations

(QC samples) from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3x the lower limit of quantification (LLOQ) (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC).

Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported.

The stability of the analyte in biological matrix at intended storage temperatures should be established. The influence of freeze-thaw cycles (a minimum of three cycles at two concentrations in triplicate) should be studied.

The stability of the analyte in matrix at ambient temperature should be evaluated over a time period equal to the typical sample preparation, sample handling, and analytical run times.

Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument failure.

The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix. For hyphenated mass spectrometry-based methods, however, testing six independent matrices for interference may not be important. In the case of LC-MS and LC-MSMS-based procedures, matrix effects should be investigated to ensure that precision, selectivity, and sensitivity will not be compromised.

Method selectivity should be evaluated during method development and throughout method validation and can continue throughout application of the method to actual study samples.

Acceptance/rejection criteria for spiked, matrix-based calibration standards and validation QC samples should be based on the nominal (theoretical) concentration of analytes. Specific criteria can be set up in advance and achieved for accuracy and precision over the range of the standards, if so desired.

2.3.2 High Performance Liquid Chromatography (HPLC) (44)

2.3.2.1 High-performance liquid chromatography (Figure 2.7)

High-performance liquid chromatography or high-pressure liquid chromatography (HPLC) is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture.

HPLC utilizes different types of stationary phase (typically, hydrophobic saturated carbon chains), a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. The detector may also provide other characteristic information (i.e. UV/Vis spectroscopic data for analyte if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase.

With HPLC, a pump (rather than gravity) provides the higher pressure required to propel the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography.

2.3.2.2 Reversed phase HPLC (RP-HPLC or RPC)

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is a silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With these stationary phases, retention time is longer for molecules which are more non-polar, while polar molecules elute more readily. An investigator can increase retention time by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent. RPC is so commonly used that it is often incorrectly referred to as "HPLC" without further specification. The pharmaceutical industry regularly employs RPC to qualify drugs before their release.

Figure 2.7 High Performance Liquid Chromatography with UV detector.

RPC operates on the principle of hydrophobic forces, which originate from the high symmetry in the dipolar water structure and play the most important role in all processes in life science. RPC is allowing the measurement of these interactive forces. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of the analysis.

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) results in a longer retention time because it increases the molecule's non-polar surface area, which is non-interacting with the water structure. On the other hand, polar groups, such as -OH, $-NH_2$, COO^- or $-NH_3^+$ reduce retention as they are well integrated into water. Very large molecules, however, can result in an incomplete interaction between the large analyte surface and the ligand's alkyl chains and can have problems entering the pores of the stationary phase.

Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C-C-bonds elute later than those with a C=C or C-C-triple bond, as the double or triple bond is shorter than a single C-C-bond.

Another important component is the influence of the pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. The buffers serve multiple purposes: they control pH, neutralize the charge on any residual exposed silica on the stationary phase and act as ion pairing agents to neutralize charge on the analyte. Ammonium formate is commonly added in mass spectrometry to improve detection of certain analytes by the formation of ammonium adducts. A volatile organic acid such as acetic acid, or most commonly formic acid, is often added to the

mobile phase if mass spectrometry is used to analyze the column eluent. Trifluoroacetic acid is used infrequently in mass spectrometry applications due to its persistence in the detector and solvent delivery system, but can be effective in improving retention of analytes such as carboxylic acids in applications utilizing other detectors, as it is one of the strongest organic acids. The effects of acids and buffers vary by application but generally improve the chromatography.

Reversed phase columns are quite difficult to damage compared with normal silica columns; however, many reversed phase columns consist of alkyl derivatized silica particles and should never be used with aqueous bases as these will destroy the underlying silica particle. They can be used with aqueous acid, but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment. RP-HPLC columns should be flushed with clean solvent after use to remove residual acids or buffers, and stored in an appropriate composition of solvent.

2.3.2.3 Isocratic flow and gradient elution

A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition). The word was coined by Csaba Horvath from Yale University who was one of the pioneers of HPLC.

The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution. (45) One example is a gradient starting at 10% methanol and ending at 90% methanol after 20 minutes. The two components of the mobile phase are typically termed "A" and "B"; A is the "weak" solvent which allows the solute to elute only slowly, while B is the "strong" solvent which rapidly elutes the solutes from the column. Solvent A is often water, while B is an organic solvent miscible with water, such as acetonitrile, methanol, THF, or isopropanol.

In isocratic elution, peak width increases with retention time linearly according to the equation for N, the number of theoretical plates. This leads to the disadvantage that late-eluting peaks get very flat and broad. Their shape and width may keep them from being recognized as peaks.

Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward. This also increases the peak height (the peak looks "sharper"), which is important in trace analysis. The gradient program may include sudden "step" increases in the percentage of the organic component, or different slopes at different times - all according to the desire for optimum separation in minimum time.

In isocratic elution, the selectivity does not change if the column dimensions (length and inner diameter) change - that is, the peaks elute in the same order. In gradient elution, the elution order may change as the dimensions or flow rate change.

The driving force in reversed phase chromatography originates in the high order of the water structure. The role of the organic component of the mobile phase is to reduce this high order and thus reduce the retarding strength of the aqueous component.

2.3.2.4 Important parameters for HPLC

Internal diameter

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column. Larger columns are usually seen in industrial applications, such as the purification of a drug product for later use. Low-ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

- Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.
- Analytical scale columns (4.6 mm) have been the most common type of columns, though smaller columns are rapidly gaining in popularity. They are used in traditional quantitative analysis of samples and often use a *UV-Vis absorbance detector*.
- Narrow-bore columns (1–2 mm) are used for applications when more sensitivity is desired either with special UV-vis detectors, fluorescence

detection or with other detection methods like liquid chromatography-mass spectrometry

- Capillary columns (under 0.3 mm) are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

Particle size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 μm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter square (46-47).

This means that changing to particles that are half as big, keeping the size of the column the same, will double the performance, but increase the required pressure by a factor of four. Larger particles are used in preparative HPLC (column diameters 5 cm up to > 30 cm) and for non-HPLC applications such as solid-phase extraction.

Pore size

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside.

Pump pressure

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 40 MPa (6000 lbf/in^2), or about 400 atmospheres. Modern HPLC systems have been improved to work at much higher pressures, and therefore are able to use much smaller particle sizes in the columns (< 2 μm). These "Ultra High Performance Liquid Chromatography" systems or RSLC/UHPLCs can work at up to 100 MPa (15,000 lbf/in²), or about 1000 atmospheres. The term "UPLC" is a

trademark of the Waters Corporation, but is sometimes used to refer to the more general technique.

2.4 Population pharmacokinetics in research by population PK program.

1.4.1 Population pharmacokinetics (25, 48-49)

Relative to the field of pharmacokinetics itself, population pharmacokinetics is in its infancy. Its origin, which can be traced to the mid-1970s, was largely the result of the efforts of Sheiner and Beal of the University of California, San Francisco (UCSF). Many more academic, industrial, and regulatory scientists have since contributed to this field. Some of the more notable events in the evolution of population pharmacokinetics have been captured in a recent review article. 1) The term population pharmacokinetics has been defined in several settings and should not be confined to pharmacokinetic responses alone, as the field, depending on the agent of interest, may encompass pharmacodynamics as well. Population pharmacokinetics can be defined as the study of the sources and correlates of variability in drug concentrations among individual who represent the target population that ultimately receives relevant dose of drug of interest. 2) Its advantages over more traditional pharmacokinetic characterization include the actual study of the population of interest (as opposed to healthy volunteers or special population only), better estimates of population means and variance, opportunities to assess multiple factors that may influence drug disposition, and at least a theoretical cost-benefit advantage. Its value as a distinct field of study can be measured indirectly from the regulatory acknowledgement provided with the recent FDA guidance on population pharmacokinetics, the increased frequency by which drug manufacturers submit population pharmacokinetic studies/analyses with their NDAs, the increased frequency in publications describing population pharmacokinetic theories and applications, and the emergence of contract research organizations (CROs) that specialize in population pharmacokinetics itself or related technologies that depend on population pharmacokinetics, such as clinical trial simulation (CTS).

The settings in which population pharmacokinetics or, more generally, nonlinear mixed effect modeling can be employed vary greatly and span all phases of drug development from discovery through phase IV. Specifically, these techniques have been employed to screen compounds for selection as drug candidates, analyze toxicokinetic data, assist with interspecies scaling, pool data from pre-clinical, non-clinical human or clinical trials as with a meta-analysis, guide special population trials, examine bioequivalence, provide guidance for dosing regimens for pivotal trials or labeling, and assist with pharmacokinetics/pharmacodynamic (PK/PD) analysis of phase I, II, III, or IV trials.

Population pharmacokinetics is the study of the sources and correlates of variability in drug concentrations among individuals who are the target patient population receiving clinically relevant doses of a drug of interest (48-49). Certain patient demographic, pathophysiological, and therapeutical features, such as body weight, excretory and metabolic functions, and the presence of other therapies, can regularly alter dose-concentration relationships. For example, steady-state concentrations of drugs eliminated mostly by the kidney are usually greater in patients suffering from renal failure than they are in patients with normal renal function receiving the same drug dosage. Population pharmacokinetics seeks to identify the measurable pathophysiologic factors that cause changes in the dose-concentration relationship and the extent of these changes so that, if such changes are associated with clinically significant shifts in the therapeutic index, dosage can be appropriately modified.

2.1.4.1 Conceptual and statistic framework (50)

Population pharmacokinetic analysis proceeds in stages. The most successful efforts have been benefit of prospective study design, clearly defined objective, and data analysis plans that are defined prior to study initiation. Although some deviation in the order of the analysis plan is possible, there is a typical sequence that is followed:

Exploratory analysis: Distribution analysis of covariates under investigation, covariate correlation analysis, and investigation into disease process time course if necessary.

Structural model definition: Comparison of all potential model representations based on available data and parameter identifiability, sensitivity analysis to determine data elements that may affect parameter identifiability (missing value, data collection errors, etc.) and simulations to estimate predictive performance prior to covariate analysis.

Parameter distribution analysis: Determination of Bayes individual parameter estimates from structural model to examine parameter covariance, estimate functional expressions of each parameter, and explore variance model expressions.

Covariate selection: Examine covariate addition to model using generalized additive modeling (GAM) or equivalent stepwise insertion techniques.

Population PK refinement: Obtain population pharmacokinetic parameter estimates, determine influential covariates and outlines, and address "reasonable" residual random error.

Validation: Determine predictive performance of the population pharmacokinetic model.

Project and application: Examine the usefulness of the population pharmacokinetic model to describe pharmacokinetic and relevant covariate relationships for regulatory submission or query, answer "real-world" questions about the data, and propose additional studies based on finding.

2.1.4.2 Individual and the Population

The very nature of research involves, at some point, the collection of data. Individual data elements are organized into common groupings to examine the diversity within and across groups. In the pharmaceutical research context, such experiments typically involve the measurement of responses of interest at several selected or randomly observed values of a related variable (or covariate). More specially, in pharmacokinetic studies we are interested in examining repeated measures (i.e., drug concentrations) over the variable time following administration of a drug to an individual animal, volunteer, or patient. Each subject in this setting belongs to a population of such individuals. The collective responses of this

population are believed to e related to the covariate in a similar manner, but with some measure of variation across individuals.

The study of pharmacokinetics has provided many techniques to analyze complex responses within an individual. From a classical statistical reference, we then seek to make inferences about the central tendency of the collective individual data. Population pharmacokinetics seeks not only to understand the mean population response profile but also to derive information about an individual, which cannot necessarily be obtained from direct measurement of repeated measures within a well-defined study. This necessitates modeling that incorporates the structural detail necessary to define the individual profile and the manner in which variation of these individual profiles exists about the mean population profile.

The history of the pharmacokinetics is laden with attempts to explain biologic processes with mathematical expressions. Current practice for the pharmacokinetic characterization of new chemical entities from well-defined, sufficiently sample studies usually involves the noncompartmental or systems analysis approach presented previously. Modeling with parameters that can be related to physiological, demographic, and pathophysiological variables can be especially important when analyzing patient data as these parameters may be useful in the quidance of initial dosing as well as maintenance regimens. Also, given the increased interest in stochastic simulation to plan future studies and examine the effects of dose and frequency on concentration-time profiles, such models (those that require structural and statistical components) are often required for the identification of patient subgroups.

2.1.4.3 Estimation methods and software

All software alternatives and methods are based on a hierarchical nonlinear mixed effects model approach described in the previous selection. The programs can be grouped into three main categories: parametric and nonparametric maximum likelihood and Bayesian. They differ in the manner in which they estimate the parameters of the nonlinear mixed effect model. Although general descriptions of each of the estimation methods are provided, a more rigorous description of these methods can be found in work of Davidian and Giltinan (51). The selection of software will necessitate a corresponding estimation method and the

subsequent assumptions and conditions that will be carried forward in the analysis. Some of these are discussed in the next subsection. In addition, the selection of software will likely dictate that the user become familiar with a pseudo-language to express the structural models exist in some packages and error models may be specified from a palate of predefined choices, the analyte will ultimately be obliged to examine more complex models requiring the mastery of fourth generation (i.e., SAS, SPLUS, Nonlin, NM-TRAN or Pred file syntax) or a formal (Visual Basic, FORTRAN) language.

Covariates may be thought of factors that explain the variability of specific parameters defined within the context of apopulation-based pharmacokinetics or pharmacodynamics model. The typically fall into demographic, pathophysiological or study-specific categories. The utility of covariates identification lies in the observation of potential differences in patient subgroup that may correlate with observed clinical outcomes, the recommendation of dose or regimen adjustment in certain subgroups based on pharmacokinetic differences, or the support for warning or precautions with dose administration in certain subgroups based on PK/PD differences in certain subpopulations. Common covariates identified in population PK/PD model such as gender, renal function, hepatic function, age, total body weight, smoking and race related with clearance (Cl) and volume of distribution (V_d).

One of the most common difficulties in the analysis of population pharmacokinetics data is that of missing data. Missing patient identifier or details of drug administration including compliance occur frequently and are best handled via prospectively defined procedures when possible. Typical solutions to this problem include case/subject deletion and imputation (inserting sample mean or median or estimation via linear regression or an EM procedure). Each of these approaches carries meth-odological requirements and potential limitations given the observed pattern of the missing data. Analytical limitations in conjunction with sample collection errors can also yield missing observation.

Strategies for handling missing values are varied and can depend on the nature and reasons for the missing information. It should be appreciated that missing value do not mean missing information and the most common and simplest approach, dropping subjects or observations, is the least informative.

Although compliance can be thought of as missing data, it can be incorporated directly into the pharmacostatistical model as either a covariate or and outcome depending on how it is estimated.

More so than traditional clinical pharmacology studies, proof-of-concept studies, or dose-finding studies, population PK/PD studies have been scrutinized because of the logistical, data accuracy, covariate assessment, and protocol design problems they present. Several meetings have been held to discuss these issue as well as specific issue relating to the design of population-based PK/PD studies. From these meeting, general guidelines have emerged:

A population PK/PD study should not compromise the main objectives of the clinical trial.

It is important to communicate the purpose of the population PK/PD analysis to the investigators and to convince them of the importance of accurate timing.

Some prior knowledge of the PK/PD models and covariate relationships is necessary for the analysis of sparse Phase III data.

Computer simulation and optimal design measures may be useful in defining sparse sampling time.

Population methods must be specified as completely as possible in the protocol.

Simulation techniques vary based on the degree of randomness desired. If one wants the typical subject only, a deterministic solution is achieved by solving the structural model for a given input at defined sampling times. Error (intra- and inter- individual) partitions are not necessary. Similarly, the extremes of a certain parameter can be simulated by fixing the estimate of that parameter to its upper or lower bound and proceeding with a deterministic solution. More informed simulations will, of course, specify the degree of stochasticity required to answer the questions posed. For example, simulation can be used during the model development and refinement process through a combination of simulation and fitting. This technique is valuable for deciding whether a model is too complex for the data collected or if a specific covariate is important for the accurate prediction of structural model parameters. In the more common setting, the fully specified model is utilized allowing

all random variables to roam within their likely limits. This will permit the most informative examination of regimen or subpopulation influence on the response.

WinNonlin® (52)

WinNonlin® is the industry standard for pharmacokinetic, pharmacodynamic and noncompartmental analysis. In addition to its extensive library of built-in PK, PD and PK/PD models, WinNonlin® supports custom, user-defined models to address any kind of data.

WinNonlin® is truly the industry standard. First released in 1984, WinNonlin® has been used by FDA since 1998. Today there are over 6000 commercial and academic WinNonlin® users in 1200 organizations worldwide. WinNonlin® is referenced in 530 publications retreivable in PubMed.

WinNonlin® accurately implements models to analyze bioequivalence based on replicated crossover studies, as required by the FDA for modified release dosage forms. Simply load your data and use the default model.

WinNonlin® provides a complete solution with data management, statistical, modeling, and visualization tools in one package. Its worksheet interface facilitates data handling and transformations. Its descriptive statistics and linear mixed effects modeling engines provide flexible pre- and post-modeling analyses. The bioequivalence wizard supports FDA standards for average, individual and population bioequivalence assessment. Additional tools enable exploration of your drug's properties through non-parametric superposition, semicompartmental modeling, deconvolution and nonparametric analysis of crossover studies. Finally, WinNonlin's chart and table wizards, and automatic chart output from modeling, produce high-quality output for your study reports. For users of other tools, WinNonlin supports data exchange with additional industry-standard analysis software, including SAS, NONMEM®, S-PLUS® and SigmaPlot®.

MONOLIX® version 2.4 (53)

MONOLIX® is a software dedicated to the analysis of nonlinear mixed effects models. It is designed for academic and industrial researchers or engineers, concerned with the analysis of longitudinal data in various fields such as

pharmacokinetics, pharmacodynamics, public health, agronomy, etc. This program is free software. It is governed by the CeCILL license under French law and abiding by the rules of distribution of free software.

The objectives of MONOLIX® are to perform:

1. Parameter estimation for nonlinear mixed effects models

- computing the maximum likelihood estimator of the population parameters, without any approximation of the model (linearization, quadrature approximation) using the Stochastic Approximation Expectation Maximization (SAEM) algorithm,

- computing standard errors for the maximum likelihood estimator

- computing the conditional modes, the conditional means and the conditional standard deviations of the individual parameters, using the Hastings-Metropolis algorithm.

2. Model selection

- computing several models using some information criteria (AIC, BIC)

- testing hypotheses using the Likelihood Ratio Test

- testing parameters using the Wald Test

3. Goodness of fit plots

4. Data simulation.

MONOLIX® also handles broad spectrum of models including models defined with differential equations, left censored data, discrete data models, repeated time to events, hidden Markov Model.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

The chemical and reagents used in this study are listed as the following:

1. Fentanyl citrate IV injection (50 μg/ml, Janssen-Cilag, Thailand)
2. Acetonitrial (HPLC grade, B&J, USA)
3. Hexane (HPLC grade, Labscan, Ireland)
4. Methanol (HPLC grade, B&J, USA)
5. Deionized Water (HPLC grade)
6. Potassium dihydrogen orthophosphate (KH_2PO_4) (Fisher, UK)
7. 85% Orthophosphoric acid (Labscan, Ireland)

3.2 Equipment

1. Vortex mixer (Gemmy Intrustrial., Taiwan)
2. HPLC with UV detector (Shimadzu, Japan)
3. Column Mightysil RP-18 GP 250 x 4.6 No. 8025506 (Kanto, Japan)
4. Centrifuge machine (Hettich Zentrifugen company, Germany)
5. pH meter (Orion, USA)
6. Micropipette (2-20 μl) (Glison, France)
7. Micropipette (50-200 μl) (Glison, France)
8. Micropipette (200-1000 μl) (Glison, France)
9. Pipette tips (2-20 μl) (Axygen, USA)
10. Pipette tips (1-200 μl) (Axygen, USA)
11. Pipette tips (200-1000 μl) (Axygen, USA)
12. Polypropylene microtube 2.0 ml (Axygen,USA)
13. Nylon membrane filter 0.45 μm (National Scientific, UK)

14. Volumetric flask (10, 25, 100, 250, 1000 ml)
15. Transfer pipette (1, 2, 3, 4, 5, 6, 10, 15, 20, 25 ml)
16. Cylinder (10, 100, 250, 1000 ml)

3.3 Methods

3.3.1 Part I: Method for bioanalysis of fentanyl

3.3.1.1 Preparation of standard solution

Stock solution of fentanyl is prepared by dissolving 50 μg/ml of fentanyl (IV injection ampule) in 10 ml deionized (DI) water. Separated solutions are prepared for calibration curve and quality control sample. Furthermore, solutions are obtained by serial dilution of stock solution with DI water.

Stock solution of internal standard (IS) is prepared by dissolving 50 mg/ml of dimenhydrinate in 100 ml of DI water and prepared 1.25 μg/ml for calibration curve and quality control samples (QC samples). All stock solutions are stored at 2 to 8 °C

3.3.1.2 Condition and sample extraction

Chemical and reagent

Fentanyl (≥ 99.5%) was kindly provided by The Department of Anesthesiologist, Phramongkutklao Army Hospital, Bangkok (50 μg/ml IV injection, Janssen-Cilag, Thailand). Dimenhydrinate (≥ 99.9%) was obtained from The Department of Medical Sciences, Ministry of Public Health of Thailand. The following HPLC grade chemicals and reagents were obtained from Science Integration Co.,Ltd.; hexane (Labscan, Ireland), acetronitrile (B&J, USA), methanol (B&J, USA) and deionized water from Central Instrument Facility, Faculty of Pharmacy, Mahidol University.

Apparatus and operating condition

The High Performance Liquid Chromatography (HPLC) system consisted of a solvent delivery module, an auto-injector port with a 500 μl of sample loop, column for separation and a UV-VIS spectrophotometric detector (Shimadzu,

Kyoto, Japan). Chromatographic separation of drugs from extracted plasma samples was performed in isocratic mode with a reverse phase C-18 column (Mightysil RP-18 GP 250 x 4.6 (5 microns) No.8025506 at ambient temperature. The mobile phase consisted of 70% of 0.05 M K_2HPO_4 buffer solution with pH adjust to 2.8, 20% acetonitrile and 10% methanol. The flow rate through the HPLC column was maintained at 1.0 ml/min and the UV absorbance was measured at 210 nm. The injection volume was 100 µl and the runtime was set at 40 min. HPLC chromatographic data were calculated by Class-VP software.

Sample preparation

The following procedure was developed to be as an extraction process for samples as well as calibrators and QC samples. Firstly, a 20 µl volume of the internal standard working solution was added to the 300 µl plasma in a 2.5 ml plastic tube and then a 50 µl of 5M NaOH, a 100 µl of acetonitrile and a 1.5 ml of *n*-hexane were added. After shaking for 1 min on a vortex shaker, samples were centrifuged at 5000 rpm for 5 min. An aliquot of clear supernatant was transferred to new plastic tube and evaporated under the hood for 3 hrs. The residue reconstituted with 300 µl mobile phase and was centrifuged at 15000 rpm in 15 min prior to injection into the analytical system.

Calibration and quality control (QC) samples

The stock solutions of fentanyl and dimenhydrinate which was used as internal standard (IS) were prepared in deionized water at a concentration of 5 µg/ml and 1.25 µg/ml, respectively. The standard solutions of fentanyl were prepared by serially diluted of the stock solution with deionized water to obtain final drug concentration of 10, 15, 20, 25, 35, 40, 50, 55, 65 and 70 ng/ml, separately. The stock solution of the IS was diluted to obtain the working solution at 1.25 µg/ml. These solutions were used to prepare calibration curve standards and quality control (QC) samples. A new set of standard solutions was prepared for each batch of samples.

Calibrators and QC samples were prepared by spiking equal volumes (20 µl) of the above working solutions of fenatnyl into 300 µl of drug-free plasma to yield the final plasma concentrations ranging from 30 - 60 ng/ml. For validation, QC samples were prepared by spiking blank plasma to obtain three levels (low, medium and high) of the final concentration at 30, 45 and 60 ng/ml, respectively.

3.3.l.3 Method development and chemical validation

The validity of the analytical method was confirmed according to the FDA guideline (36) for bioanalytical method validation by investigating selectivity, linearity, accuracy, precision, recovery and stability of analytes. All statistical tests were performed using Microsoft Excel 2007 software.

The method development and establishment phase defines the chemical assay. The fundamental parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Measurements for each analyte in the biological matrix should be validated. In addition, the stability of the analyte in spiked samples should be determined. Typical method development and establishment for a bioanalytical method include determination of 1) selectivity and sensitivity, 2) accuracy, precision, recovery, 3) calibration curve, and 4) stability of analytes in spiked samples.

A. Selectivity and sensitivity

Pooled-blank plasma samples were analyzed according to the procedure previously described in order to evaluate method specificity. The absence of interfering compounds, characterized by peak area ratio equal to those of the investigated analytes and eluting at the same analytes retention time, was verified.

Sensitivity was assessed by determining the lower limit of quantification (LLOQ) of fentanyl. The LLOQ was established as the lowest concentration of fentanyl used in the calibration curve with accuracy and precision of 100% ± 20% and signal to noise ratio > 5. Bias (%R.E.) (Equation 1) and relative standard deviation (%R.S.D.) (Equation 2) were used as measures of accuracy and precision, respectively.

$$\%\ \text{R.E.}^{a} = \frac{(\text{theoretical concentration} - \text{mean observed concentration})}{\text{theoretical concentration}} \times 100 \qquad \textit{Eq.1}$$

$$\%\ \text{R.S.D.}^{b} = \frac{\text{S.D.}}{\bar{X}} \times 100 \qquad \textit{Eq.2}$$

Where S.D. = Standard deviation

$\bar{X}$ = Mean value of analyzed drug concentration in spiked sample

[a] Percentage of relative error

[b] Percentage of relative standard deviation (%R.S.D.) or % coefficient of variation (%CV)

B. Calibration curve

Calibration curves were constructed by linear regression of the peak area ratio of fentanyl to IS (Y-axis) against the nominal standard fentanyl concentration (X-axis). Concentrations of QC samples were calculated using the equation of the calibration curve. Each of calibrator was analyzed in duplicate. The calibration curve with the coefficient of determination (R^2) > 0.9 was acceptable. Back-calculated calibration concentrations were also determined, as well as the accuracy and precision of the calibration standards, i.e. the percentage deviation of the so-determined concentrations from nominal ones (%R.E.) and the relative standard deviation (%R.S.D.), respectively.

C. The Precision and Accuracy

The precision of the assay procedure was assessed from the percent relative standard deviation values (%R.S.D.) of analyzed drug concentration in spiked plasma samples. Three difference concentrations of fentanyl in plasma were prepared and used to determine within-run (intra-day) and between-run (inter-day) precision. For within-run precision, five replicates of each concentration were assayed within on one day whereas between-run precision was performed by assaying the samples of each concentration for three consecutive days.

A minimum of three concentrations in the range of expected concentrations is recommended. The %CV determined at each concentration level, should not exceed 15 % except for the LLOQ, where it should not exceed 20 %.

The accuracy of the analytical method was calculated as the percent relative error in the calculated mean concentration relative to the nominal fentanyl concentration (%R.E.). For the assay to be considered acceptable, the precision and accuracy determined at each QC concentration level was required to be within 15%.

D. Extraction recovery

The extraction recovery of the assay was evaluated for fentanyl at low, medium and high concentrations (n = 6). The absolute recovery at each concentration was evaluated by comparing the peak area ratio of fentanyl to IS in

plasma after extraction to the standard solution containing the same concentration of fentanyl and IS in mobile phase. The percentage of recovery was calculated as the ratio of the peak area ratio of fentanyl to IS in plasma after extraction to that of the standard solution. A good recovery should be more than 90%.

The relative recovery of the analyte in this assay was determined by comparing peak area of the analyte obtained from the extracted QC plasma sample to that of the extracted blank plasma spiked with the pure authentic standard. The absolute recovery and relative recovery followed Equation 3 and 4.

$$\text{The absolute recovery} = \frac{\text{Peak area ratio of fentanyl and IS extracted from plasma}}{\text{Peak area ratio of unextracted fentanyl and IS}} \times 100 \qquad \textit{Eq.3}$$

$$\text{The relative recovery} = \frac{\text{Peak area ratio of fentanyl and IS extracted from plasma}}{\text{Peak area ratio of fentanyl and IS that spike in extracted blank plasma}} \times 100 \qquad \textit{Eq.4}$$

E. Stability

The stability of fentanyl in plasma at concentrations of 30 and 60 ng/ml was evaluated by determining the short-term stability (12 hour at room temperature, 25 ± 5°C), freeze and thaw stability (three freeze-thaw cycles) and long-term stability (3 months at -20°C). The post-preparative stability of the analyte (24 hour in the autosampler of the HPLC) was also studied. The concentrations were compared to the corresponding results before storage.

1. Short-term temperature stability at room temperature was evaluated by spiking plasma with fentanyl at two different concentrations (30 and 60 ng/ml). The samples were kept at room temperature for 12 hour. After that, the sample were spiked with IS, extracted and analyzed.

2. Freeze and thaw stability was evaluated by spiking plasma with fentanyl at two different concentrations (30 and 60 ng/ml). The samples were frozen for 24 hour at -20°C then allowed to thaw unassisted at room temperature. This process was repeated two more times, and after the third cycle, samples were spiked

with IS, extracted and analyzed. The samples were analyzed together with a freshly prepared calibration curve.

3. Long-term stability of fentanyl in plasma was evaluated by spiking plasma with fentanyl at two different concentrations (30 and 60 ng/ml). The samples were kept at -20°C for 3 months. After that, the sample were spiked with IS, extracted and analyzed.

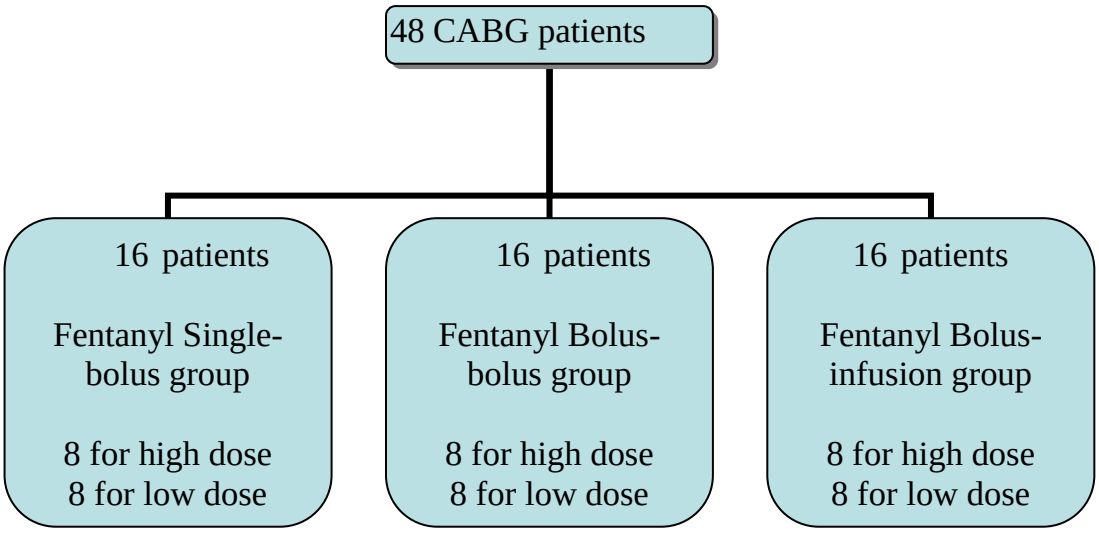
4. The post-preparative stability was also studied. QC samples from the first day were kept on the autosampler of the HPLC at room temperature for 24 hour and injected again. Fentanyl was quantified using the calibration curve constructed on the day of analysis.

5. Stock solution stability is demonstrated by preparing a fresh solution from the reference material and comparing the absolute response of the fresh solution with that of the stored solution. The acceptable difference between the absolute responses of fresh stock solutions and aged stock solutions should be within 5%.

3.3.2 Part II: Study design : Pharmacokitic data analysis.

3.3.2.1 The study design

A double blind randomized controlled trial, 48 patients were separated in 6 groups (8 persons / group). Subjects are randomized to high or low dose.



High dose = 50 mcg/kg , Low dose = 10 mcg/kg

3.3.2.2 Subjects

Forty-eight patients are recruited in the study based on the follow criteria.

Inclusion criteria

1. Patients, confirmed by physician through physical examination, medical history and laboratory test such as American Society of Anesthesiologist (ASA)[a] status II-IV , have baseline preoperative mean arterial pressure not over 100 mmHg , pulse rate between 50-80 times/min , serum creatinine not more than 1.9 mg%.

2. Male and Female subjects between the age of 18-70 years with the body mass index (BMI) not over 30 kg/m^2.

3. Do not bypass graft before and throughout the study.

4. Do not take steroid before and throughout the study.

5. Agree with the study and sign the written inform consent.

Exclusion criteria

1. Do not accept inform consent.

2. Patients are in ASA status I or V.

3. History of bypass graft or other types of heart surgery.

4. History of emergency bypass graft.

[a] American Society of Anesthesiologist (ASA) : The ASA physical status classification system is a system for assessing the fitness of patients before surgery. In 1963 the American Society of Anesthesiologists (ASA) adopted the five-category physical status classification system; a sixth category was later added. These are:

1. A normal healthy patient.

2. A patient with mild systemic disease.

3. A patient with severe systemic disease.

4. A patient with severe systemic disease that is a constant threat to life.

5. A moribund patient who is not expected to survive without the operation.

6. A declared brain-dead patient whose organs are being removed for donor purposes.

3.3.2.3 Drug administrations

This study is carried out with the approval of the Committee on Human Rights Related to Human Experimentation, Phramongkutklao Army Hospital, Bangkok, Thailand.

After fasting overnight or not less than 8 hours, subjects are separated in three groups. (8 patients/group)

First group (16) : administered fentanyl single bolus.

- Low dose (8) : 10 mcg/kg
- High dose (8) : 50 mcg/kg

Second group (16) : administered fentanyl bolus and infusion.

- Low dose (8) : bolus 5 mcg/kg and infusion 5 mcg/kg in rate 1 mcg/kg/hr by syringe pump.
- High dose(8):bolus 25 mcg/kg and infusion 25 mcg/kg in rate 5 mcg/kg/hr by syringe pump.

Third group (16) : administered fentanyl bolus and bolus.

Low dose (8) : 5 mcg/kg and 5 mcg/kg before use CPB.

High dose (8) : 25 mcg/kg and 25 mcg/kg before use CPB.

Note: CPB=Cardio Pulmonary Bypass

Administration of fentanyl in each group, was calculated by body weight of each patients and was divided by 3 major groups.

Group I and II : an anesthesiologist loaded fentanyl in single bolus at 15 minute before sternotomy.

Group III and IV : an anesthesiologist divided fentanyl into 2 equal doses. First, bolus-dosing pushed 15 minute before sternotomy and second bolus dose when started to use heart-lung machine.

Group V and VI : an anesthesiologist divided fentanyl into 2 equal doses. First, bolus-dosing pushed 15 minute before sternotomy and second infusion dose when started to use heart-lung machine in calculate infusion rate. A HPLC Class-VP with auto-injector, interfaced with UV detector, was used to determine fentanyl plasma concentration.

3.3.2.4 Collection plasma sample

Ten milliliters of blood was withdrawed from heparinized catheter prior to drug administration (baseline), 5 minute after opened sternum bone , before used cardio-pulmonary bypass (heart-lung machine), 5 minute after used cardio-pulmonary bypass machine, between used cardio-pulmonary bypass every 60 minute (2 – 3 times depend on surgery procedure), post cardiopulmonary bypass, skin closured and 24 hour after fentanyl dosing. (about ten points per patient, at least seven points) Then, the whole blood was centrifuged at 5000 rpm for 10 min at room temperature for blood plasma separation. The blood plasma is separated and kept at stable temperature (-80°C) until assay.

3.3.2.5 Pharmacokinetic data analysis

The pharmacokinetic analysis : compartmental and noncompartmental analysis will be used to determine each individual pharmacokinetic parameters using WinNonlin® Professional Edition Version 3.1.

For noncompartmental analysis, the plasma concentration time profile in each subject is fitted using noncompartmental model of extravascular input in WinNonlin®. The λ_z is calculated according to the log-linear portion of terminal phase of plasma concentration time course. The time range for determining λ_z is selected to get log-linearity. The AUC_{tlast} is calculated using log-linear trepazoid. If possible AUC from time zero to infinity is extrapolated using equation below.

$$AUC_{0\text{-}INF} = AUC_{tlast} + \frac{C_{last}}{\lambda_z} \qquad Eq.5$$

where C_{last} (ng/ml) is the last observable plasma concentration is each subject.

For compartmental analysis, all data parts obtained in each subjects will be fitted based on appropriate model for individual dosage regimen. The equation 6 is used for I.V.bolus and/or the equation 7 is used for I.V. infusion.

$$C_p(t) = \frac{Dose}{V_d} * e^{-k_e * t} \qquad Eq.6$$

$$C_p(t) = Css * (1 - e^{-ke*t}) = \frac{Dose / \tau}{k_e * V_d} (1 - e^{-k_e t}) \qquad Eq.7$$

where $C_p(t)$ (ng/ml) is plasma concentration of drug at anytime (t),

Dose (mcg/kg) is administered dose,

V_d is volume of distribution,

k_e is elimination rate constant (hr^{-1})

C_{ss} is plasma concentration of drug at steady state which can be calculated from (Dose / τ) / (k_e * V_d) where τ is infusion duration.

3.3.2.6 Population pharmacokinetic and statistical data analysis

Parameter obtained from each dosage regimen in patients will be compared by using one-way analysis of variance (1-way ANOVA) or appropriate statistical analysis by Monolix® program. The level of significance was taken at p value of 0.05. Monolix® program was estimated correlation between fentanyl pharmacokinetic parameters and appropriated covariate values.

CHAPTER IV
RESULTS

4.1 High Performance Liquid Chromatography

4.1.1 Method validation

4.1.1.1 Method optimization

The composition and flow rate of mobile phases were optimized for HPLC-UV system. An achieved system could separate fentanyl, midazolam and IS within 22 min. The total chromatographic run time was less than 40 min. Retention times of fentanyl and IS were 16.35 ± 0.05 and 21.85 ± 0.05 min (mean ± S.D.), respectively.

4.1.1.2 Selectivity and the limit of quantification (Figure 4.1)

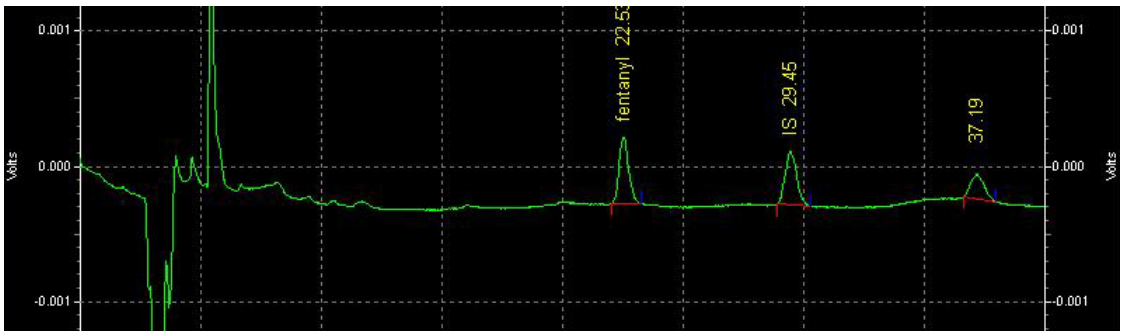
It was shown that there was no endogenous from blood plasma interfering peaks appeared at the retention time of fentanyl (16 min) and IS (22 min).

Sensitivity was assessed by determining the LLOQ of fentanyl. The LLOQ for fentanyl in plasma samples was established at 10 ng/ml with a precision (% R.S.D.) and accuracy (%bias) of 5.11% and 2.86%, respectively as shown in Table 4.1.

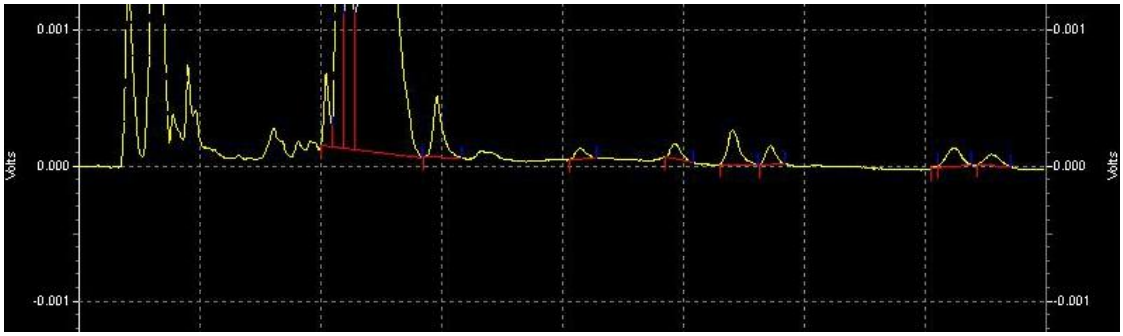
4.1.1.3 Calibration curve

Calibration curves were constructed by linear regression of the peak area ratio of fentanyl to IS (Y-axis) against the nominal standard fentanyl concentration (X-axis). The calibration curve was linear over the range of 10-70 ng/ml and the correlation coefficient (R^2) was > 0.9 for all validation batches. Figure 4.2 and Table 4.2 shows a typical calibration curve for the fentanyl assay. Calibration standards concentrations were back calculated %R.S.D. and %Bias. %R.S.D. and %Bias value was from 0.17% to 12.93% and |-14.17|% to |14.38|%, respectively for all concentration levels.

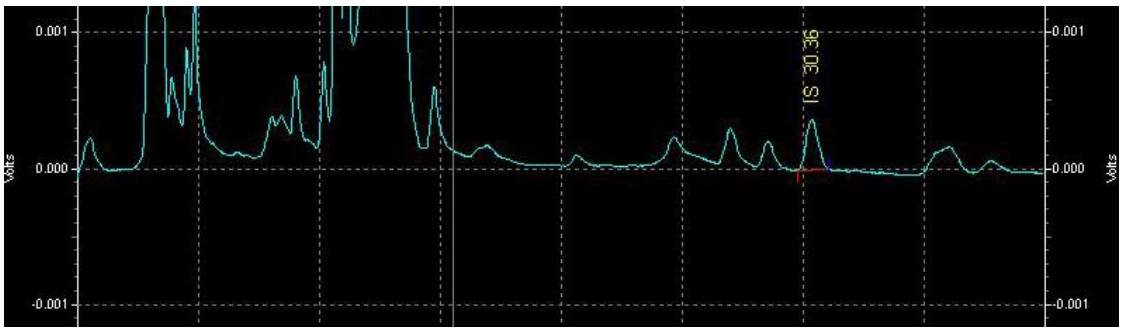
Reference standard



Blank plasma



Blank zero plasma



QCM sample

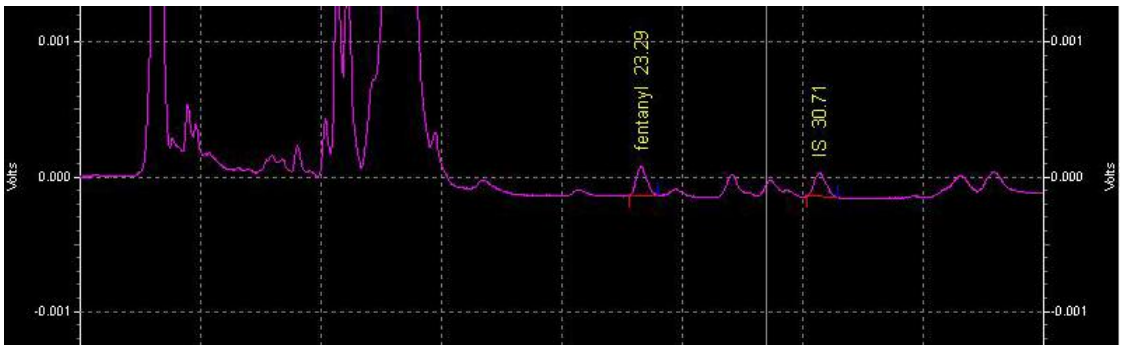


Figure 4.1 The chromatogram of reference standard (fentanyl and dimenhydrinate), blank plasma, zero blank plasma and QCM sample.

4.1.1.4 The precision and accuracy

The precision of the system was investigated by means of repeatability and reproducibility from three QC samples (30, 45 and 60 ng/ml). The accuracy of the analytical method was determined by five replicate injections of three different concentrations of the standard solution of fentanyl that spike in blood sample. Intra- and inter-day accuracy were less than |10.51| and |6.77|% respectively, while intra- and inter-day precision was less than 12.21 and 5.27%, respectively. The results were shown in the Table 4.3.

4.1.1.5 Extraction recovery

The extraction recovery of the assay was evaluated for fentanyl at low, medium and high concentrations (n = 6). The absolute recovery at each concentration was calculated by ratio of peak area between fentanyl and IS in extracted plasma divided by the ratio of standard solution containing the same concentration of fentanyl and IS in mobile phase. The relative extraction recovery showed in Table 4.4.

4.1.1.6 Stability

The stability of fentanyl in plasma at concentrations of 30 and 60 ng/ml was evaluated by determining the short-term stability, freeze and thaw stability and long-term stability. The post-preparative stability of the analyte was also studied. All data of stability showed in Table 4.5-4.7.

The stability data of fentanyl in the matrice are shown in Table 4.5. The amount of fentanyl remaining after all these tests was found to be more than 92.68%. In other words, fentanyl was determined to be stable under different temperature and storage conditions.

For the post-preparative stability, the data of fentanyl and IS was shown in Table 4.6. In each QC sample pool, the concentrations were determined on the basis of original calibration standards. The mean analyzed results were within 15% of the nominal sample concentrations and the precision for the replicate analysed not exceed 15%.

The stock solution of fentanyl was stable for at least 1 month when the solution was kept in a refrigerator at 4°C. (Table 4.7)

It can be seen from the above results that the HPLC method used in this study demonstrated a good precision and accuracy which was suitable used for analysis fentanyl concentration in collected blood sample of cardio-pulmonary bypass graft patients.

4.1.2. The Example how to determinate fentanyl concentration in collected samples of CABG patients.

The method was applied to analyse plasma samples obtained from cardio-pulmonary bypass graft patients. A run was rejected if more than a third of QC sample concentrations showed a deviation from the theoretical concentration equal to or greater than 20%.

The example of procedure was patient number 45 and 46 as shown in Table 4.8. A calibration curve of peak area ratio (Y-axis) versus the ten concentrations of fentanyl (X-axis) was presented in Figure 10. The linear regression equation of the standard curve was calculated to be: $Y = 0.0355X - 0.1751$ with a good coefficient of determination (R^2) of 0.9955. The fentanyl concentration in the sample solution was calculated from the equation of the standard curve. The concentration of fentanyl in QC samples was shown in Table 4.9.

Table 4.1 The lower limit of quantification

LLOQ	Fentanyl concentration (ng/ml)						Mean ± S.D.	R.S.D. (%)	R.E. (%)
	1	2	3	4	5	6			
10	10.20	9.79	9.56	9.98	9.96	8.80	9.71 ± 0.50	5.11	2.86

Table 4.2 The theoretical and mean observed concentration of calibration curve of fentanyl by HPLC method

Theoretical concentration (ng/ml)	Mean observed concentration [a] ± S.D. (ng/ml)	R.S.D. (%)	R.E. (%)
10	11.42 ± 1.48	12.93	-14.17
15	16.49 ± 0.03	0.17	-9.95
20	17.12 ± 0.26	1.54	14.38
25	26.04 ± 2.01	7.70	-4.15
35	39.68 ± 6.23	5.69	-13.37
40	33.63 ± 1.74	5.17	5.92
50	51.80 ± 6.48	12.52	-3.60
55	49.46 ± 1.41	2.84	10.08
65	62.54 ± 1.24	1.98	3.78
70	76.04 ± 6.54	8.61	-8.62

[a] $n = 2$

$Y = 0.0231X - 0.1407$, $R^2 = 0.9632$

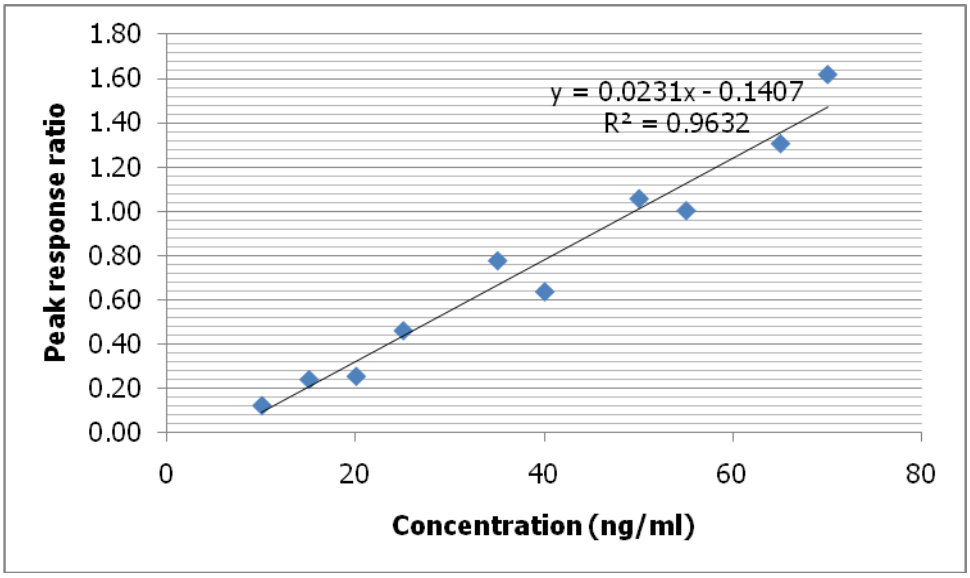


Figure 4.2 A calibration curve of fentanyl and internal standard ratio (10-70 ng/ml) for the determination of fentanyl concentrations in sample solutions by HPLC method.

Table 4.3 Accuracy and precision of fentanyl by HPLC method

Nominal concentration (ng/ml)	Mean observed concentration ± S.D. (ng/ml)	Precision [c] (%)	Accuracy [d] (%)
Intra-day 1 [a]			
30	28.34 ± 2.09	7.37	6.77
45	42.54 ± 4.64	10.90	5.55
60	60.90 ± 6.98	11.47	-1.61
Intra-day 2 [a]			
30	29.01 ± 1.05	3.63	3.30
45	46.15 ± 5.63	12.21	-2.56
60	66.31 ± 3.38	5.10	-10.51
Intra-day 3 [a]			
30	28.64 ± 1.70	5.92	4.53
45	43.16 ± 2.08	4.82	4.08
60	60.35 ± 1.47	2.44	-0.57
Inter-day [b]			
30	28.66 ± 0.34	1.17	4.87
45	43.95 ± 1.93	4.39	2.36
60	62.52 ± 3.29	5.27	-4.23

[a] $n = 5$

[b] $n = 15$; three similar determinations ($n = 5$) on different days

[c] Percentage of relative standard deviation (%R.S.D.)

[d] Percentage of difference between mean observed and nominal concentrations (%R.E.)

Table 4.4 The relative recovery of fentanyl by HPLC method

Analytes	Concentration [a] (ng/ml)	Relative recovery Mean (%) ± S.D.(ng/ml)	%R.S.D
Fentanyl	30	132.18 ± 6.00	5.85
	45	137.86 ± 7.66	1.44
	60	138.49 ± 3.36	0.46
Dimenhydrinate	1250	84.16 ± 4.52	1.98

[a] $n = 6$

Table 4.5 The stability of spike human plasma at low and high QC samples

Nominal concentration[a] (ng/ml)	Mean observed concentration ± S.D. (ng/ml)	R.S.D. (%)	R.E. (%)
Short-term stability (25°C, 12 h)			
30	29.96 ± 0.34	1.15	0.14
60	63.16 ± 4.22	6.69	-5.27
Freeze-thaw stability (3 cycle)			
30	28.97 ± 8.72	9.03	3.44
60	59.66 ± 4.61	7.73	0.56
Long-term stability (-20°C, 3 months)			
30	27.80 ± 1.30	4.69	7.31
60	58.86 ± 2.39	4.07	1.89

[a] $n = 3$

Table 4.6 The post-preparative stability

Nominal concentration [a] (ng/ml)	Mean observed concentration ± S.D. (ng/ml)	R.S.D. (%)	R.E. (%)
h_o			
30	31.84 ± 3.41	10.71	-6.15
45	47.07 ± 3.54	7.52	-4.59
60	62.95 ± 4.31	0.07	-4.92
h_{24}			
30	30.13 ± 3.45	11.47	-0.43
45	47.51 ± 2.36	4.96	-5.57
60	65.06 ± 4.31	0.07	-8.43

[a] $n = 3$

Table 4.7 Stock solution stability

Day	Fentanyl Peak Area [a] (5000 ng/ml) Mean ± SD	R.S.D (%)	Dimenhydrinate Peak Area (1250 ng/ml) Mean ± SD	R.S.D (%)
0	30046 ± 267	0.89	9665 ± 172	1.78
7	20308 ± 250	1.23	8966 ± 162	1.81
30	28565 ± 217	0.76	8840 ± 126	1.43

[a] $n = 3$

Table 4.8 The example data of standard curve used to analysis fentanyl concentration in CPBG patients

Concentration (ng/ml)	Peak area of fentanyl	Peak area of dimenhydrinate	Peak area ratio
10	883	5766	0.15
15	1951	5161	0.38
20	2328	4241	0.55
25	3076	4067	0.76
35	6121	6158	0.99
40	5971	4928	1.21
50	9321	5850	1.59
55	8328	4461	1.87
65	11149	5142	2.17
70	12578	5596	2.25

Table 4.9 The QC samples of this batch. (Patient no. 45 and 46)

Times	Peak area of fentanyl	Peak area of IS	Peak area ratio	Concentration of fentanyl (ng/ml)	R.E. [b] (%)	Pass [c]
Low QC [a]						
1	5018	6175	0.81	27.82	7.26	Pass
2	4037	4972	0.81	27.80	7.32	Pass
Medium QC						
1	7654	4800	1.59	49.85	-10.78	Pass
2	6590	5650	1.17	37.79	16.03	Not pass
High QC						
1	9930	5477	1.81	56.00	6.66	Pass
2	10039	4217	2.38	71.99	-19.98	Not pass

[a] Low QC (30 ng/ml), Medium QC (45 ng/ml), High QC (60 ng/ml)

[b] %R.E. (relative error) was calculated from

$$\frac{(\text{Nominal concentration} - \text{Experiment concentration})}{\text{Nominal concentration}} \times 100$$

[c] more than 4/6 = Pass

$Y = 0.0355X - 0.1751$, $R^2 = 0.9955$

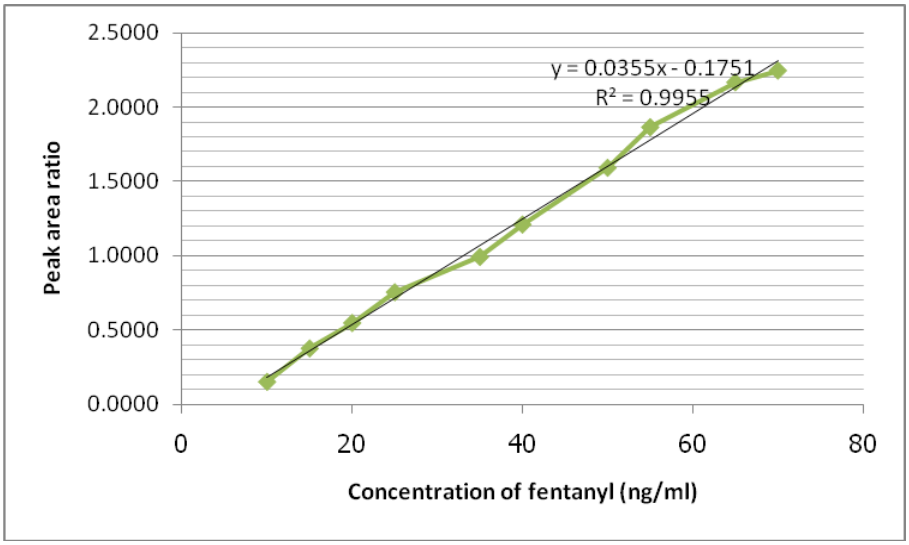


Figure 4.3 A calibration curve of fentanyl for the determination of fentanyl concentrations in patient 45 and 46.

4.2 The data of drug analysis.

4.2.1 The study design.

A double blind randomized controlled trial, 48 patients were separated in 6 groups (8 persons / group). Subjects are randomized to high or low dose followed by chart in Figure 2.4 and Table 4.3-4.4 showed all patient characteristics.

The averaged weight, 62.8 kilograms and the averaged age, 58.2 years was incorporated into the model after centering.

4.2.2 Blood sampling data

Fourty-eight adults patient with inclusion criteria were enrolled in this study. When the vital signs had stabilized, fentanyl in each dosing type was administered. Ten milliliters of blood was withdrawed from heparinized catheter prior to drug administration (baseline), 5 minute after opened sternum bone , before used cardio-pulmonary bypass (heart-lung machine), 5 minute after used cardio-pulmonary bypass machine, between used cardio-pulmonary bypass every 60 minute (2-3 times depend on surgery procedure), post cardiopulmonary bypass, skin closured and 24 hour after fentanyl dosing. (ten points per patient, at least seven points)

The time samplings in each group, the concentration of fentanyl and the data points were detected showed in Table 4.5, 4.6 and 4.7, respectively.

Table 4.10 A randomized controlled trial, 48 patients separates in 6 groups

Groups	Dosing	Patient No.
Single-bolus group	High dose (50 mcg/kg)	1 7 15 21 29 35 41 46
	Low dose (10 mcg/kg)	6 14 20 28 32 36 47 48
Bolus-bolus group	High dose (25-25 mcg/kg)	5 11 13 19 27 34 37 42
	Low dose (5-5 mcg/kg)	4 10 12 18 26 33 38 43
Bolus-infusion group	High dose (25 mcg/kg-5 mcg/kg/hr)	3 9 17 23 25 31 39 44
	Low dose (5 mcg/kg-1 mcg/kg/hr)	2 8 16 22 24 30 40 45

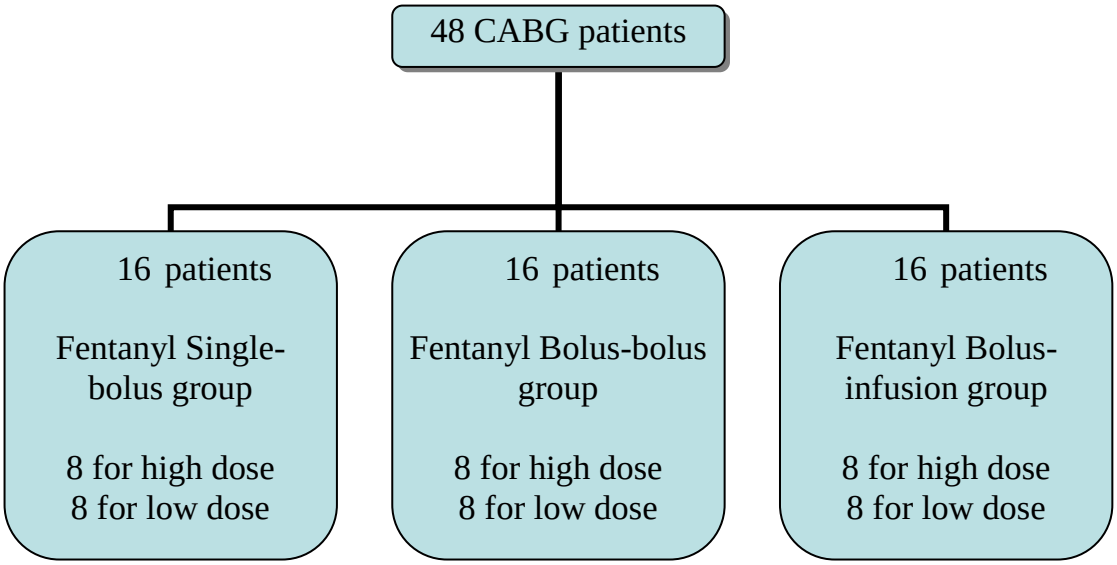


Figure 4.4 The flow chart of CABG patients in 6 groups.

Table 4.11 Patient characteristics

Types	Gr. I [a]	Gr. II	Gr. III	Gr. IV	Gr. V	Gr.VI
Genders (M :F)	5:3	8:0	5:3	6:2	7:1	7:1
Age (Year ± S.D.)	58.50 ± 5.24	59.00 ± 6.39	57.87 ± 11.59	60.12 ± 6.56	58.75 ± 6.61	56.37 ± 9.07
Weight (Kg ± S.D.)	58.62 ± 9.42	66.75 ± 8.05	65.37 ± 11.49	70.00 ± 7.35	67.62 ± 9.96	64.62 ± 11.50
Height (cm ± S.D.)	161.50 ± 6.07	164.00 ± 2.24 [b]	156.75 ± 6.23	165.5 ± 7.84	164.62 ± 4.14	165.12 ± 5.08

[a] $n = 8$

[b] Gr.II $n = 7$

Table 4.12 The time samplings of each group

Group I ; Single-bolus group : high dose (50 mcg/kg)

ID	Time of sampling (hour : min)									
	1	2	3	4	5	6	7	8	9	10
001	13:35	14:33	15:17	15:50	16:50	17:50	18:10	18:37	19:37	12:00
007	13:33	13:57	14:47	15:15	16:10	16:30		16:45	17:15	
015	8:45	9:05	10:05	10:10	11:00	11:15		11:30	11:50	17:00
021	8:05	9:00	9:40	10:00	10:55	11:35		11:55	12:20	13:00
029	11:55	12:55	13:25	13:50	14:50	15:05		15:18	15:40	13.25
035	11:30	12:30	13:05	13:10	14:10	15:15		15:25	15:45	15:30
041	12:20	14:00	14:30	14:55	15:55			16:55	17:30	16:53
046	9:40	12:00	13:15	13:30	15:15	16:25	16:30	17:00	17:45	15:15

Table 4.12 The time samplings of each group (continued)

Group II ; Single-bolus group : low dose (10 mcg/kg)

ID	Time of sampling (hour : min)									
	1	2	3	4	5	6	7	8	9	10
006	8:30	9:20	9:38	11:05	11:55	13:00	13:38	14:30	12:50	11:50
014	8:15	9:25	9:55	10:15	11:00	11:45		12:00	12:20	11:00
020	8:30	9:20	10:00	10:35	11:30	12:30		12:50	13:40	13:20
028	14:25	15:40	16:27	16:55	17:50	18:50	19:30	19:55	20:30	14:45
032	12:00	12:25	13:00	13:14	14:10	15:10	15:25	15:45	16:40	17:10
036	12:00	12:50	13:30	13:55	14:55	15:20		15:30	15:50	15:10
047	9:40	10:15	11:10	12:10	13:10	14:10		15:12	16:30	17:30
048	12:05	13:15	13:45	14:25	15:20	16:10		16:30	17:10	

Table 4.12 The time samplings of each group (continued)

Group III ; Bolus-bolus group : high dose (25-25 mcg/kg)

ID	Time of sampling (hour : min)									
	1	2	3	4	5	6	7	8	9	10
005	8:30	9:20	9:38	11:05	11:55	13:00	13:38	13:40		12:50
011	13:05	14:40	15:20	15:45	16:45	17:45	18:00	18:20	19:10	16:30
013	10:00	12:15	12:45	13:05	14:00			14:12	14:35	15:35
019	8:30	9:50	10:15	10:45	11:40			12:25	12:50	14:30
027	11:05	12:55	13:35	14:00	15:00	15:35		15:45	16:10	15:00
034	14:25	15:30	16:15	16:45	17:45	18:30		18:55	19:05	15:15
037	11:00	12:05	12:35	13:10	14:10	15:10	15:25	15:45	16:00	N/A
042	8:30	9:50	10:47	11:45	12:35	13:25		13:45	14:45	16:15

Table 4.12 The time samplings of each group (continued)

Group IV ; Bolus-bolus group : low dose (5-5 mcg/kg)

ID	Time of sampling (hour : min)									
	1	2	3	4	5	6	7	8	9	10
004	10:42	11:37	12:15	12:50	13:45	14:45	15:10	15:45	16:45	
010	8:35	9:10	9:20	9:48	10:30			11:10	11:35	8:30
012	12:30	13:10	13:30	14:00	15:00	15:40		16:05	16:45	16:00
018	14:28	15:20	16:20	16:51	17:45	18:45	19:45	19:55	20:45	16:30
026	14:20	14:55	15:50	16:35	17:30	18:30	19:20	20:00	20:50	14:30
033	8:15	9:15	10:35	10:55	11:55	12:20		13:00	13:40	15:15
038	12:40	13:40	14:15	14:35	15:45	16:40		17:00	17:20	15:00
043	8:00	9:15	10:11	10:30	11:30	11:45		12:05	12:25	14:00

Table 4.12 The time samplings of each group (continued)

Group V ; Bolus infusion group : high dose (25 mcg/kg-5 mcg/kg/hr)

ID	Time of sampling (hour : min)									
	1	2	3	4	5	6	7	8	9	10
003	10:25	11:35	11:50	12:03	12:43			13:05	13:25	12:20
009	11:50	12:45	13:15	14:35	15:40	16:00	16:30	16:45	17:30	12:00
017	14:15	15:30	16:25	16:40	17:40	18:15		18:45	19:20	
023	13:10	14:55	16:10	17:00	18:00	19:00	19:40	20:05	21:20	14:30
025	8:00	9:15	9:45	10:13	11:06	11:45		12:05	12:30	13:30
031	12:45	14:42	15:00	15:15	16:15	17:15	17:32	17:55	18:55	16:00
039	7:35	9:00	9:40	10:35	11:35	12:35	12:55	13:10	14:00	15:30
044	8:20	10:55	12:25	13:10	14:10	15:10		15:40	16:25	14:00

Table 4.12 The time samplings of each group (continued)

Group VI ; Bolus infusion group : low dose (5 mcg/kg-1 mcg/kg/hr)

ID	Time of sampling (hour : min)									
	1	2	3	4	5	6	7	8	9	10
002	11:20	11:20	13:15	13:25	14:25	15:25		16:22	16:50	12:40
008	8:35	9:15	9:20	9:35	9:45	10:50		11:05	11:20	16:30
016	12:45	13:28	14:05	14:30	15:20	16:20		16:30	17:10	16:00
022	14:35	15:35	16:00	16:55	17:55	18:55		19:35	20:10	14:20
024	15:25	16:25	16:50	17:20	18:20	19:20	19:20	19:50	20:40	15:55
030	15:10	15:50	16:25	16:55	17:55	18:55	19:50	20:05	20:40	
040	8:45	9:20	9:58	10:25	11:25	12:00		13:00	13:30	15:00
045	12:10	14:55	15:24	15:45	16:40	17:00		17:10	17:40	14:00

Table 4.13 The concentration of fentanyl in each sampling point

Group I : Single bolus group : high dose (50 mcg/kg)

ID	Concentration (ng/ml)									
	1	2	3	4	5	6	7	8	9	10
001	0	22.96	15.08	0	0	0	0	0	0	0
007	14.28	42.21	17.94	10.32	0	0		0	0	
015	0	17.54	19.94	25.11	18.77	18.44		14.52	13.43	0
021	0	12.90	10.16	0	0	0		0	0	0
029	0	24.06	15.52	18.00	10.12	10.36		0	0	0
035	0	34.27	24.95	336.23	15.78	12.57		0	13.30	0
041	0	27.85	19.06	13.12	13.20			11.46	0	0
046	0	18.19	13.68	0	13.63	0	0	0	0	0

0 = Below Quantitative Limited (BQL=10 ng/ml)

Table 4.13 The concentration of fentanyl in each sampling point (continued)

Group II : Single bolus group : low dose (10 mcg/kg)

ID	Concentration (ng/ml)									
	1	2	3	4	5	6	7	8	9	10
006	0	181.40	26.12	11.31	0	24.91	0	0	0	0
014	0	0	0	0	0	0		0	0	0
020	0	0	0	13.98	0	0		0	0	0
028	0	0	0	0	0	0	0	0	0	0
032	0	0	0	0	0	0	0	0	0	0
036	0	11.22	0	0	0	0		0	0	0
047	0	0	0	0	14.37	0		0	0	0
048	0	0	14.89	0	0	16.36		0	0	

0 = Below Quantitative Limited (BQL=10 ng/ml)

Table 4.13 The concentration of fentanyl in each sampling point (continued)

Group III : Bolus bolus group : high dose (25-25 mcg/kg)

ID	Concentration (ng/ml)									
	1	2	3	4	5	6	7	8	9	10
005	10.79	0	11.63	11.68	0	0	0	0		11.24
011	0	11.09	80.45	13.74	11.23	0	0	0	0	0
013	0	12.25	65.85	20.12	11.38			11.31	0	0
019	137.54	20.79	18.57	13.05	0			0	0	0
027	0	11.58	14.84	0	0	11.03		11.31	12.60	0
034	0	126.10	35.12	16.25	14.40	15.55		16.41	12.41	14.24
037	0	12.41	22.65	0	0	0	0	0	0	0
042	0	0	17.44	15.23	0	0		0	0	0

0 = Below Quantitative Limited (BQL=10 ng/ml)

Table 4.13 The concentration of fentanyl in each sampling point (continued)

Group IV : Bolus bolus group : low dose (5-5 mcg/kg)

ID	Concentration (ng/ml)									
	1	2	3	4	5	6	7	8	9	10
004	0	0	12.70	0	0	0	0	0	0	
010	0	0	19.84	0	0			0	0	0
012	0	0	0	0	0	0		0	0	0
018	0	0	10.61	0	0	0	0	0	0	0
026	0	0	0	0	0	0	0	0	0	0
033	0	0	0	12.48	0	0		0	0	0
038	0	0	0	0	0	0		0	0	0
043	0	0	0	0	0	0		0	0	0

0 = Below Quantitative Limited (BQL=10 ng/ml)

Table 4.13 The concentration of fentanyl in each sampling point (continued)

Group V : Bolus infusion group : high dose (25 mcg/kg-5 mcg/kg/hr)

ID	Concentration (ng/ml)									
	1	2	3	4	5	6	7	8	9	10
003	0	0	11.13	0	0			0	22.60	0
009	0	0	0	0	0	0	0	0	0	0
017	0	22.33	14.63	11.89	N/A	0		0	13.21	
023	0	10.61	0	0	0	0	0	0	0	0
025	0	14.39	0	0	0	0		11.69	15.20	0
031	37.62	0	0	12.40	0	0	12.39	0	0	0
039	0	16.46	0	0	0	13.57	0	12.43	12.74	0
044	0	0	0	0	0	0		0	0	0

0 = Below Quantitative Limited (BQL=10 ng/ml)

Table 4.13 The concentration of fentanyl in each sampling point (continued)

Group VI : Bolus infusion group : low dose (5 mcg/kg-1 mcg/kg/hr)

ID	Concentration (ng/ml)									
	1	2	3	4	5	6	7	8	9	10
002	0	0	0	0	0	0		0	0	0
008	0	0	0	0	0	0		0	0	0
016	0	13.79	0	0	0	0		0	0	0
022	0	0	0	0	0	0		0	0	0
024	0	0	0	0	0	0	0	0	0	0
030	0	0	0	0	0	0	0	0	0	
040	0	0	0	0	0	0		0	0	0
045	0	0	0	0	0	10.21		0	0	0

0 = Below Quantitative Limited (BQL=10 ng/ml)

Table 4.14 The data points were detected

Group [a]	No. patient all (persons)	No. patient detect (persons)	No. sampling Points	No. detecting Point	detecting (%)
Group I	8	8	72	34	47.2
Group II	8	5	74	9	12.2
Group III	8	8	72	34	47.2
Group IV	8	4	73	4	5.5
Group V	8	6	74	17	23.0
Group VI	8	2	73	2	2.7

[a] The six group of dosage regimen consists of

Group I : Single-bolus group-high dose (50 mcg/kg)

Group II : Single-bolus group-low dose (10 mcg/kg)

Group III : Bolus-bolus group-high dose (25-25 mcg/kg)

Group IV : Bolus-bolus group-low dose (5-5 mcg/kg)

Group V : Bolus-infusion group-high dose (25 mcg/kg-5 mcg/kg/hr)

Group VI : Bolus-infusion group-low dose (5 mcg/kg-1 mcg/kg/hr)

The mean of duration and standard deviation of operation procedure was 310 ± 72 min. Pharmacokinetic analysis of this research used two programs : Winnonlin® program for estimated the initial values such as volume of distribution (V_d) and clearance (Cl) and Monolix® program for estimated the covariate results about age, body weight and dose typing in two compare dosing groups. The sex cannot be estimate in this study because some of group had only one type of sex. The plasma concentration time profile in each subject is fitted using noncompartment model in sparse model of Winnonlin® as shown in Table 4.15.

Winnonlin® sparse model will extrapolate all of data points in a pair of groups such as 1) Single-bolus : Group I and II : between high and low dosing group which compared the level of dosing in the same dosing type. 2) Group I and III : high dosing group: Single-bolus and Bolus-bolus which compared two dosing type in same dosing. 3) Group II and IV : low dosing group : Single-bolus and Bolus-bolus which compared two dosing type in same dosing. The others did not estimate with sparse model because there has a few data points.

All of the initial values are important for fixing data of volume of distribution and clearance in Monolix® program. If there is no an initial value, the data will not fit in an appropriate model for individual dosage regimen.

For the single-bolus group : Group I (high dose) and Group II (low dose) were estimated by Monolix® program. Parameters were compared by using one-way analysis of variance (1 way ANOVA). The level of significance was taken by p-value of 0.05. The analysis results are shown in Figure 4.5-4.11 and Table 4.16-4.17.

Table 4.15 The initial values from sparse model of Winnonlin® in a pair of group

Value	Volume of distribution (L)	Clearance (ml/min)
Between High and low dosing : Single bolus group (I and II)	86	0.055
Between Single-bolus and Bolus-bolus : High dosing group (I and III)	156	0.39
Between Single-bolus and Bolus-bolus : Low dosing group (II and IV)	35	0.058

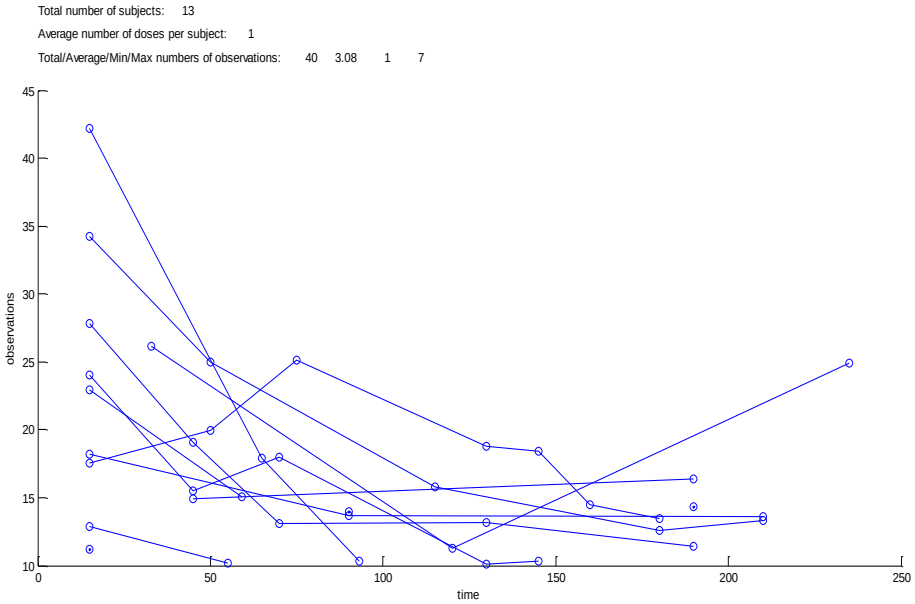


Figure 4.5 The spaghetti plot of group I and II, compare high and low group in single bolus.

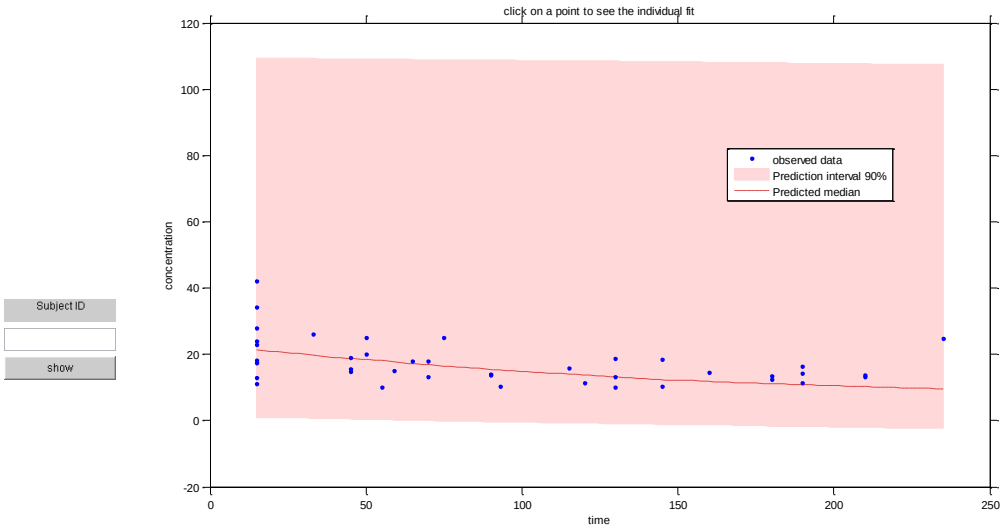


Figure 4.6 Observed data and prediction interval of group I and II. Graph shows mean with 90% prediction intervals of simulated concentrations in the pink interval. And the red line was the prediction median.

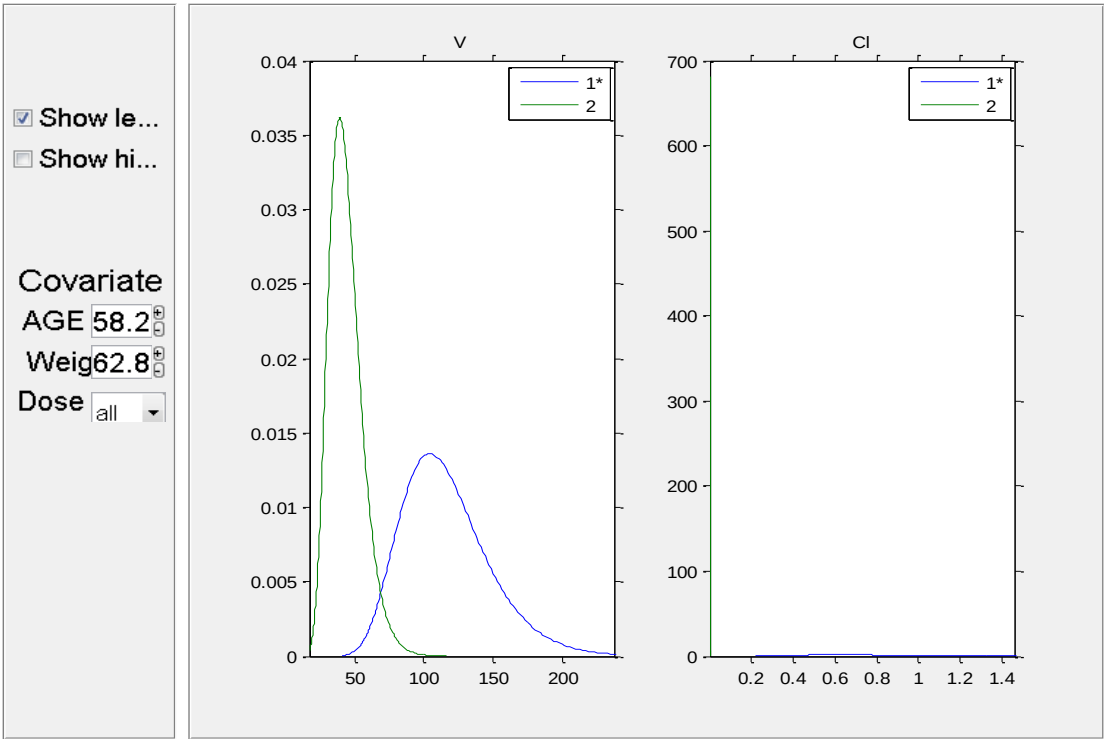


Figure 4.7 Volume of distribution and Clearance with mean age and mean weight covariate. (group I and II). The data of parameters showed in Table 4.16.

Table 4.16 Volume of distribution and Clearance of Single-bolus : high-low dosing group

Parameters	V_d (L) Mean ± S.D.	Cl (ml/min) Mean ± S.D.
Group I	117.0 ± 32.4	0.690 ± 0.206
Group II	43.8 ± 12.2	0.0022 ± 0.000656

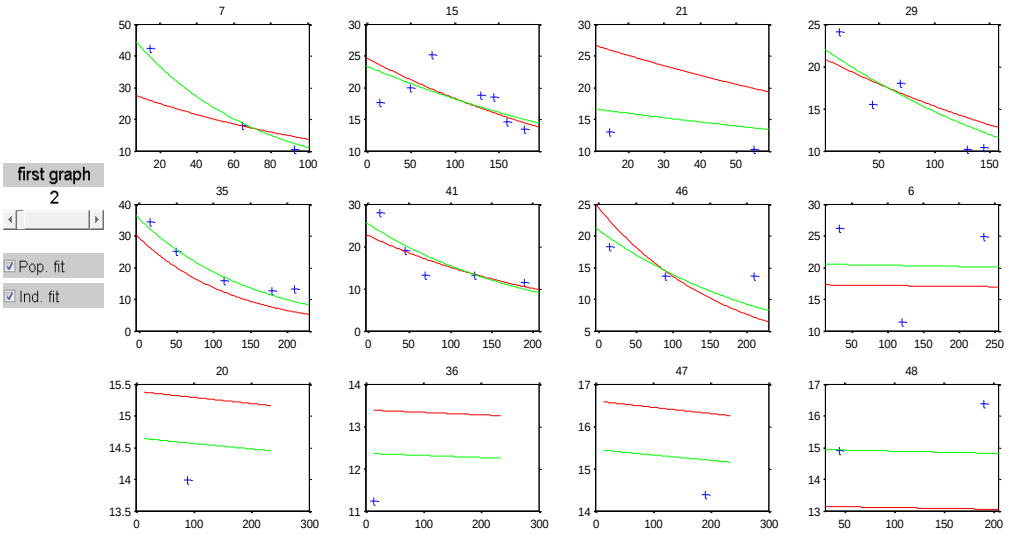


Figure 4.8. The individual fit that estimate by Monolix® program. (group I and II) The green line represented the individual fit and the red line represented the population fit.

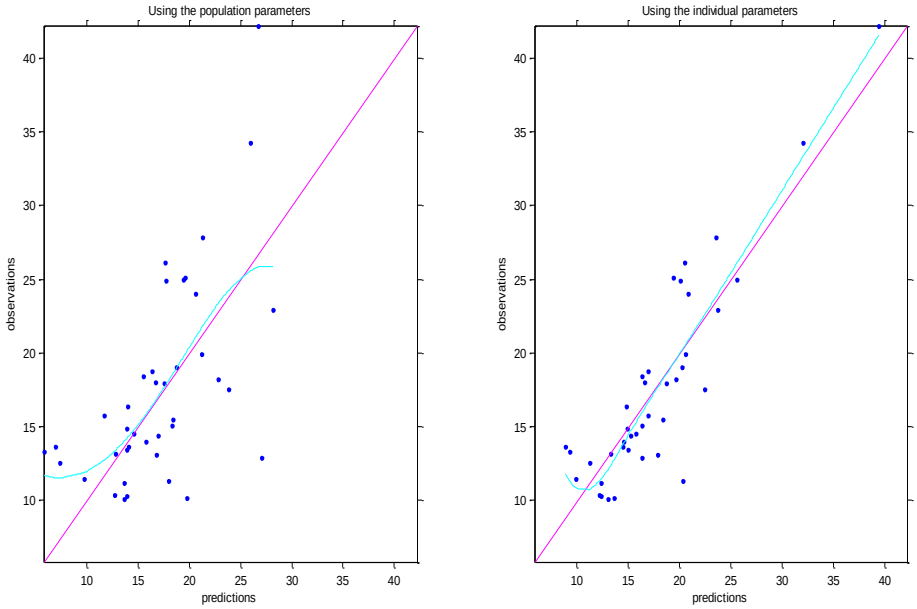


Figure 4.9 Goodness of fit plot for the observed and prediction concentrations in using population parameters (left) and using individual parameters (right) of group I and II. Solid lines are the line of unity.

The model adequately characterized the observed concentrations (Figure 4.9). A large variability between pop predicted vs. observed plasma fentanyl concentrations was significantly reduced in the plot of individual predicted vs. observed concentrations. Individual predicted concentration demonstrated good distribution around the line of unity.

The box plots of covariate and parameters showed in Figure 4.10 and 4.11. The graph showed the log volume of distribution and clearance of group I was more than group II.

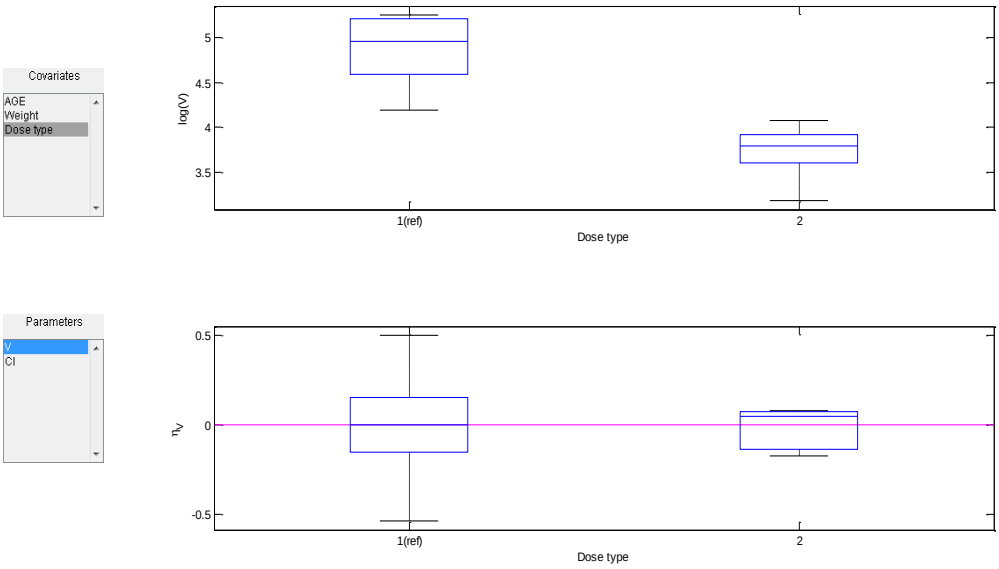


Figure 4.10 Box plot of Dose type and Volume of distribution. (group I and II)

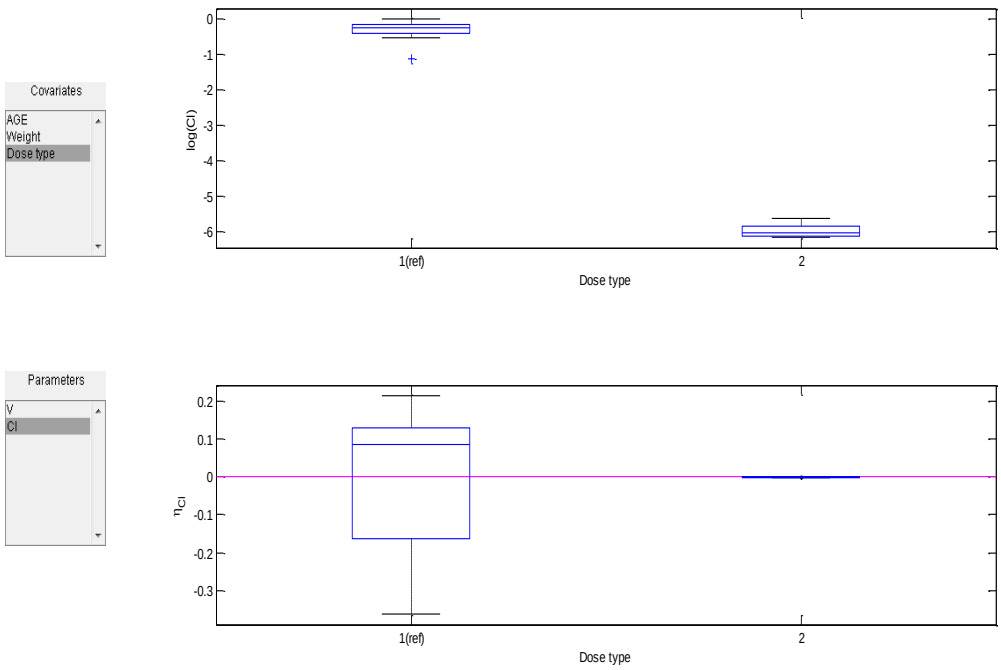


Figure 4.11 Box plot of Dose type and Clearance. (group I and II)

Table 4.17 Estimation of the population parameters. (Single-bolus: high-low dosing group)

	parameter	s.e. (lin)	r.s.e (%)	p-value
V_d	52.4	17	147	
beta_V (age)	-0.0152	0.02	135	0.46
beta_V (weight)	0.0262	0.01	40	*0.012*
beta_V (dose type_2)	-0.9790	0.22	23	*8.96-006*
Cl	0.014	0.04	263	
beta_Cl (age)	0.0571	0.037	61	0.099
beta_Cl (weight)	0.00843	0.02	233	0.67
beta_Cl (dose type_2)	-5.750	12	212	0.64

Log-likelihood estimation by importance sampling

Sampling distribution for the random effects: t with 5 d.f.

-2 X log-likelihood: 241.80 (0.037)

Akaline Information Criteria (AIC): 263.80 (0.037)

Bayesian Information Criteria (BIC): 270.02 (0.037)

The parameters of this group were evaluated in population pharmacokinetic. If the parameters are significant at $p < 0.05$, covariate values were significant related. In group I and II, the volume of distribution was significant associated with body weight. Moreover group II had significantly difference of V_d between group I.

For high dose group : Group I and Group III (between Single-bolus and Bolus-bolus) were estimated by Monolix® program. Parameters would be compared by using one-way analysis of variance (1 way ANOVA). The level of significance was taken by p-value of 0.05. The analysis results are shown in Figure 4.12-4.18 and Table 4.18-4.19.

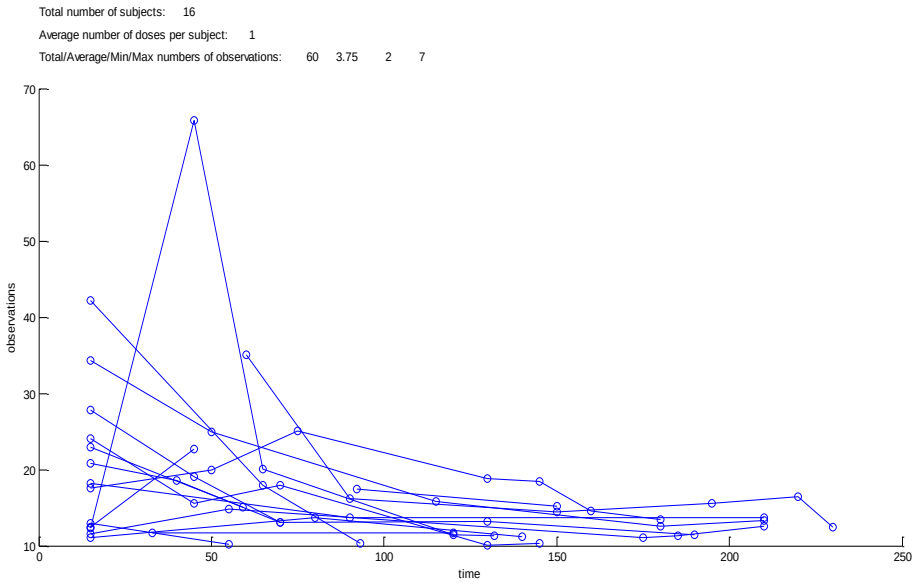


Figure 4.12 The spaghetti plot of group I and III, high dosing group compared the single-bolus and bolus-bolus group.

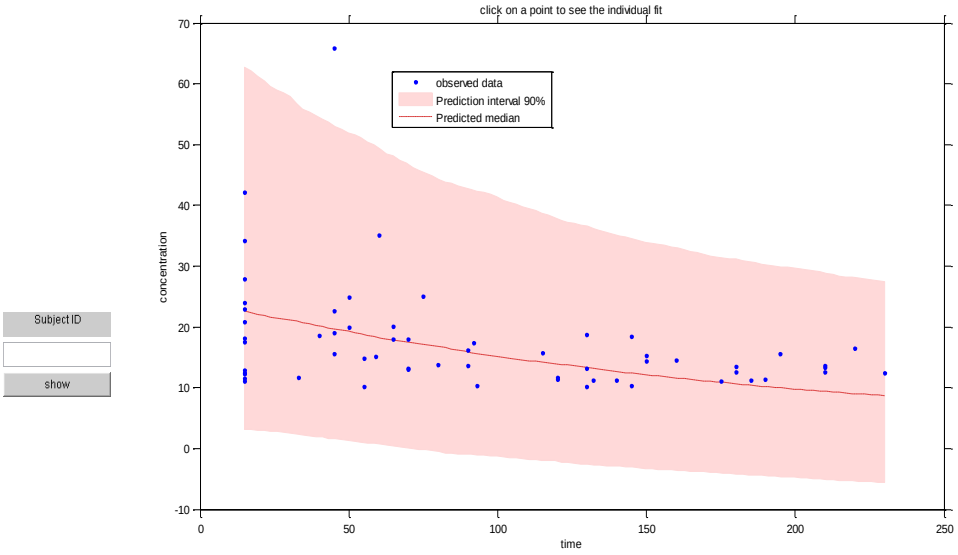


Figure 4.13 Observed data and prediction interval of group I and III. Graph shows mean with 90% prediction intervals of simulated concentrations in the pink interval. And the red line was the prediction median.

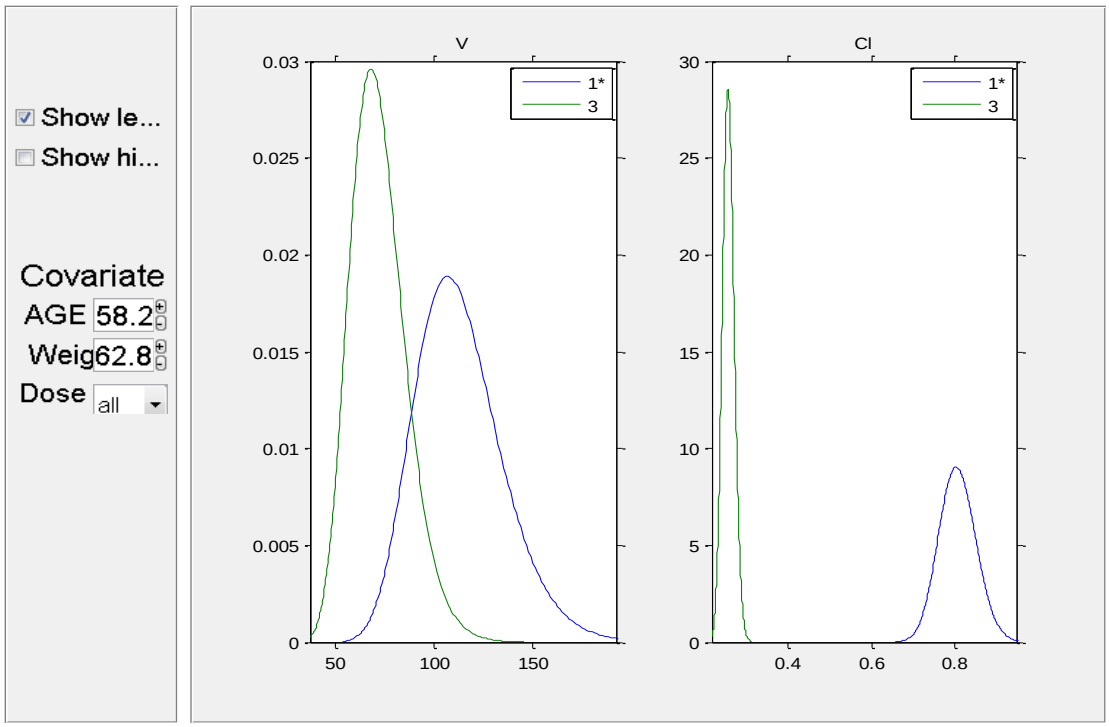


Figure 4.14 Volume of distribution and Clearance with mean age and mean weight covariate. (group I and III). The data of parameters showed in Table 4.18.

Table 4.18 Volume of distribution and Clearance of high dosing group (single-bolus and bolus-bolus)

Parameters	V_d (L) Mean ± S.D.	Cl (ml/min) Mean ± S.D.
Group I	113 ± 22.2	0.807 ± 0.0443
Group III	72.4 ± 14.2	0.255 ± 0.014

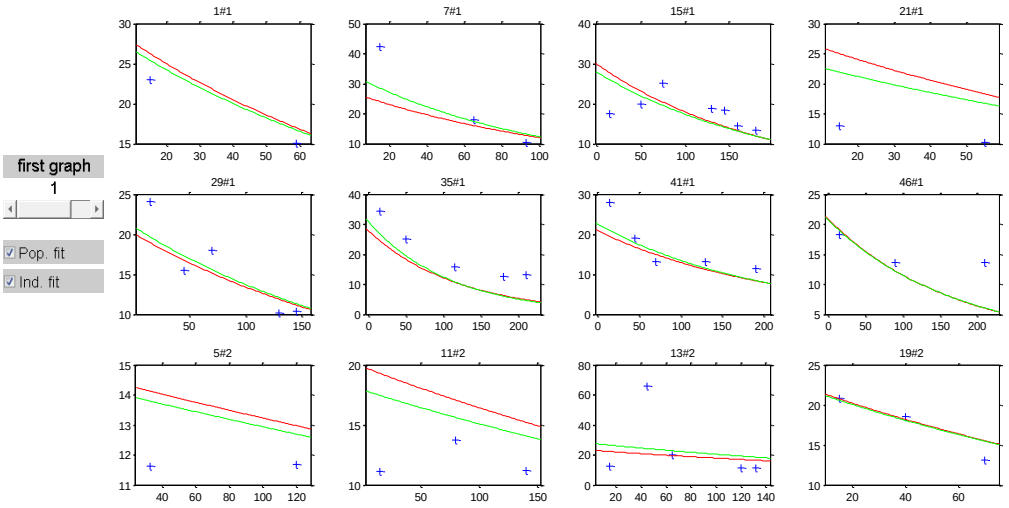


Figure 4.15 The individual fit that estimate by Monolix® program. (group I and III) The green line represented the individual fit and the red line represented the population fit.

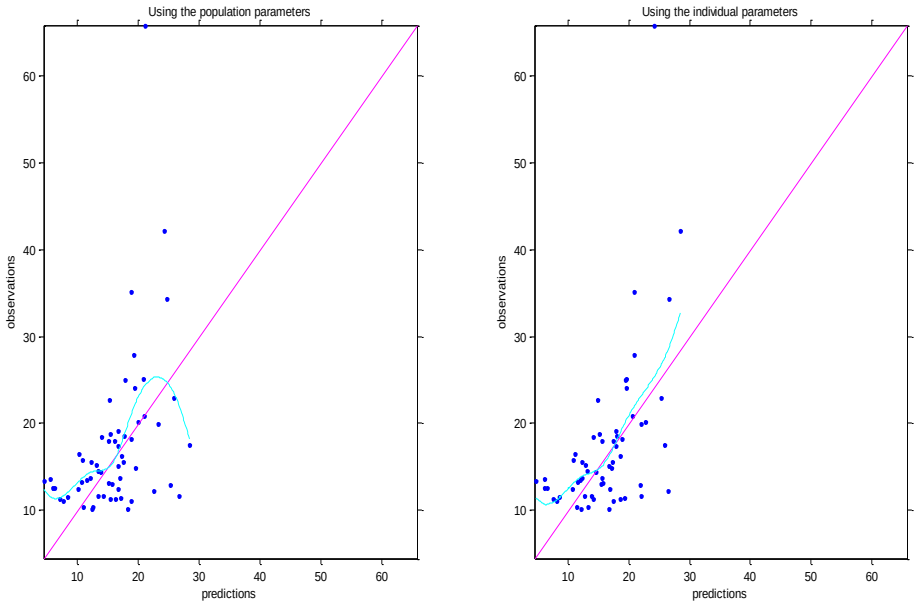


Figure 4.16 Goodness of fit plot for the observed and prediction concentrations in using population parameters (left) and using individual parameters (right) of group I and III. Solid lines are the line of unity.

The model adequately characterized the observed concentrations (Figure 4.16). A same variability between pop predicted vs. observed plasma fentanyl concentrations Individual predicted concentration demonstrated distribution around the line of unity as same as pop prediction.

The box plots of covariate and parameters showed in Figure 4.17 and 4.18. The graph showed the log volume of distribution and clearance of group I was more than group III.

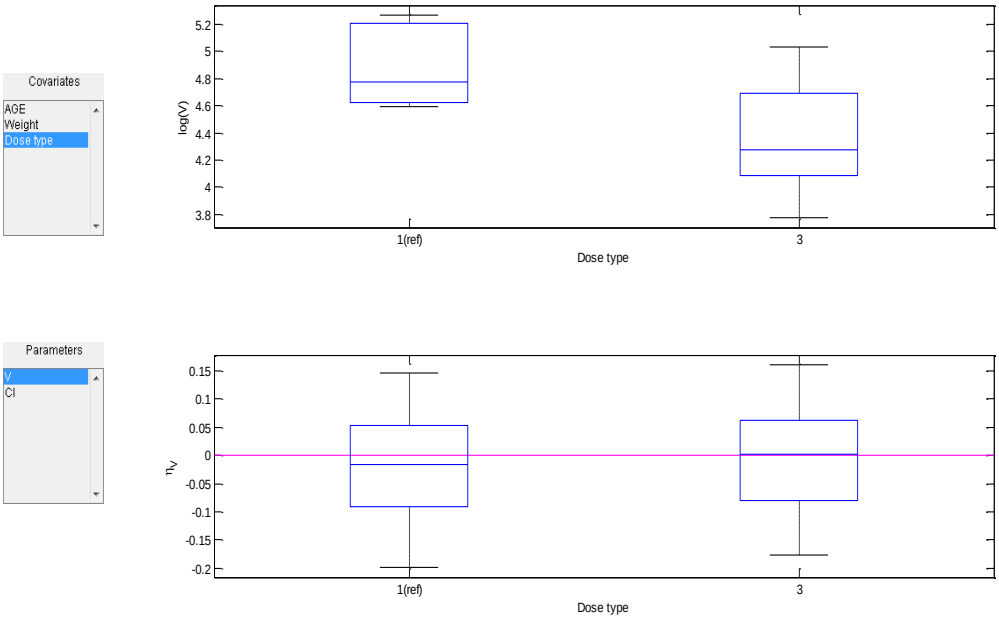


Figure 4.17 Box plot of Dose type and Volume of distribution. (group I and III)

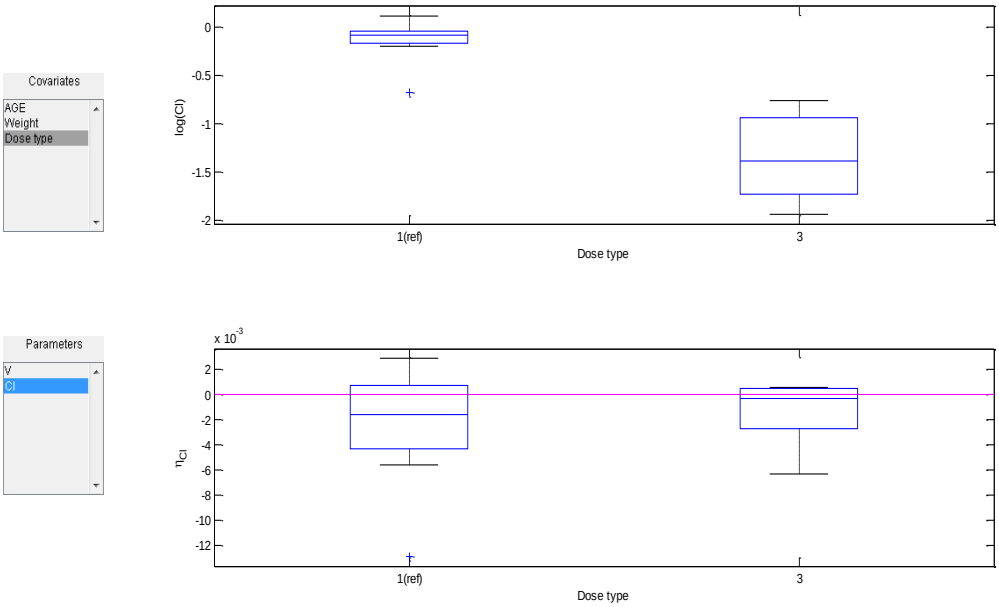


Figure 4.18 Box plot of Dose type and Clearance. (group I and III)

Table 4.19 Estimation of the population parameters with high dose : (single-bolus and bolus-bolus group)

	parameter	s.e. (lin)	r.s.e (%)	p-value
V	13.4	17	124	
beta_V (age)	0.000874	0.013	1.44e+003	0.94
beta_V (weight)	0.0328	0.012	36	*0.0056*
beta_V (dose type_3)	-0.449	0.21	46	*0.029*
	parameter	s.e. (lin)	r.s.e (%)	p-value
Cl	0.0364	0.088	240	
beta_Cl (age)	0.042	0.029	68	0.14
beta_Cl (weight)	0.0104	0.024	230	0.66
beta_Cl (dose type_3)	-1.15	0.44	38	*0.009*

Log-likelihood estimation by importance sampling

Sampling distribution for the random effects: t with 5 d.f.

-2 X log-likelihood: 424.54 (0.033)

Akaline Information Criteria (AIC): 446.54 (0.033)

Bayesian Information Criteria (BIC): 455.04 (0.033)

The parameters of this group were evaluated in population pharmacokinetic. If the parameters are significant at $p < 0.05$, covariate values were significant related. In group I and III, the volume of distribution was significant associated with body weight and dose III. Moreover group III had significantly difference of clearance between group I.

For low dosing group : Group II and Group IV (between Single-bolus and Bolus-bolus) were estimated by Monolix® program. Parameters would be compared by using one-way analysis of variance (1 way ANOVA). The level of significance was taken by p-value of 0.05. The analysis results are shown in Figure 4.19-4.25 and Table 4.20-4.21.

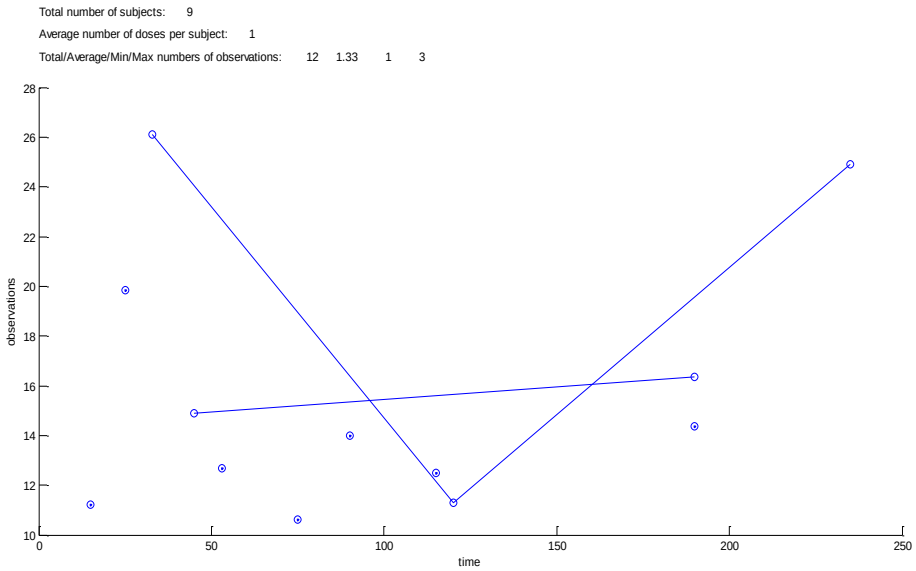


Figure 4.19 The spaghetti plot of group II and IV, low dosing group compared the single-bolus and bolus-bolus group.

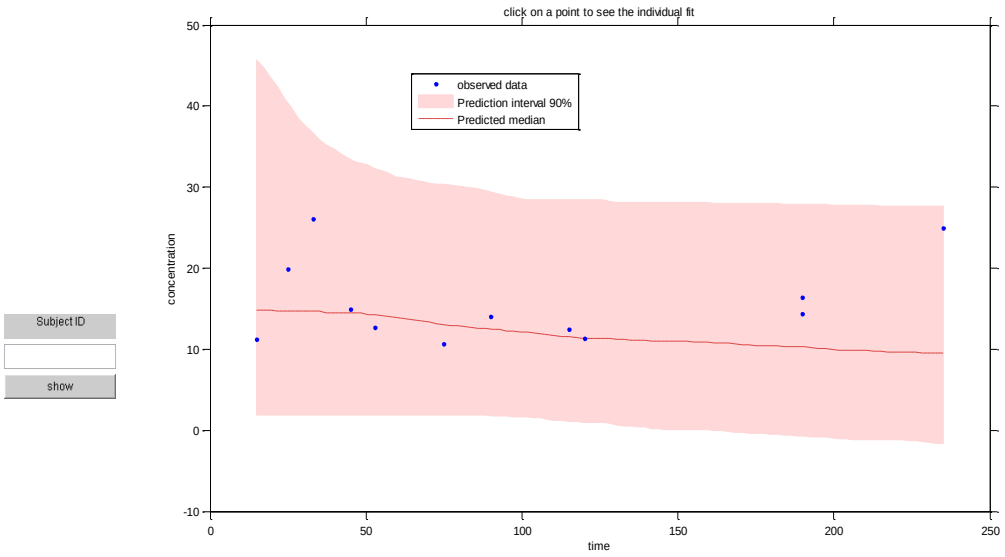


Figure 4.20 Observed data and prediction interval of group II and IV. Graph shows mean with 90% prediction intervals of simulated concentrations in the pink interval. And the red line was the prediction median.

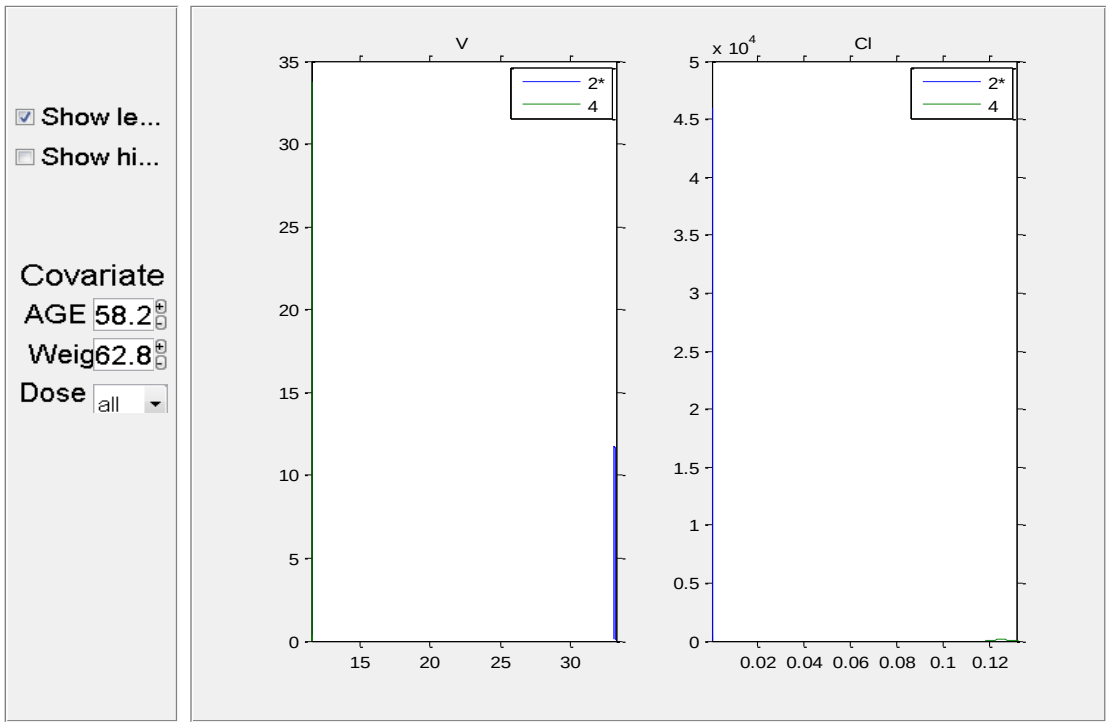


Figure 4.21 Volume of distribution and Clearance with mean age and mean weight covariate. (group II and IV). The data of parameters showed in Table 4.20.

Table 4.20 Volume of distribution and Clearance of low dosing group. (single-bolus and bolus-bolus)

Parameters	V_d (L) Mean ± S.D.	Cl (ml/min) Mean ± S.D.
Group II	33.1 ± 0.0338	0.000546 ± 8.68e-006
Group IV	11.6 ± 0.0118	0.126 ± 0.002

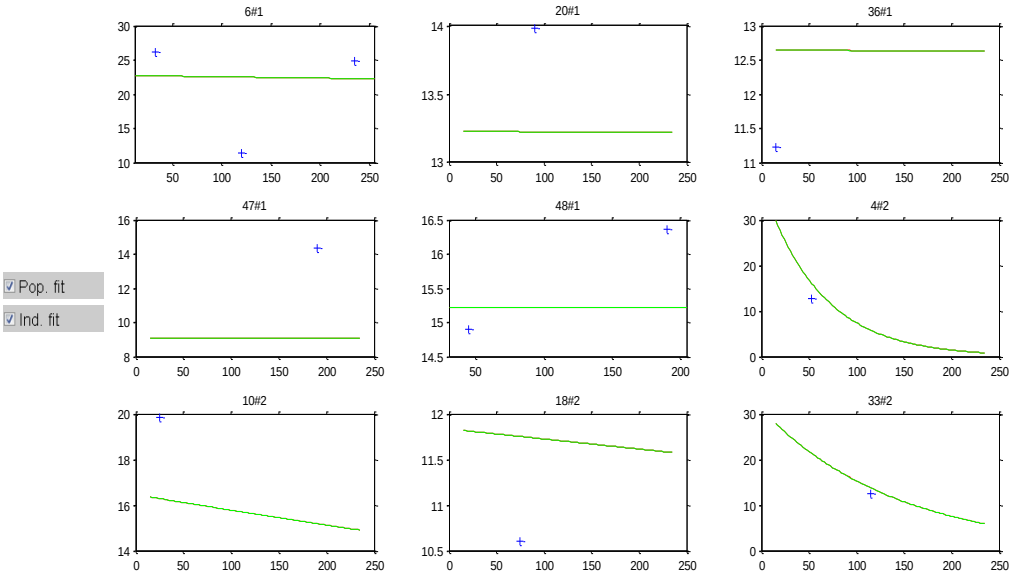


Figure 4.22 The individual fit that estimate by Monolix® program. (group II and IV) The green line represented the individual fit and the red line (not see the red line in any individual patient), which represented the population fit.

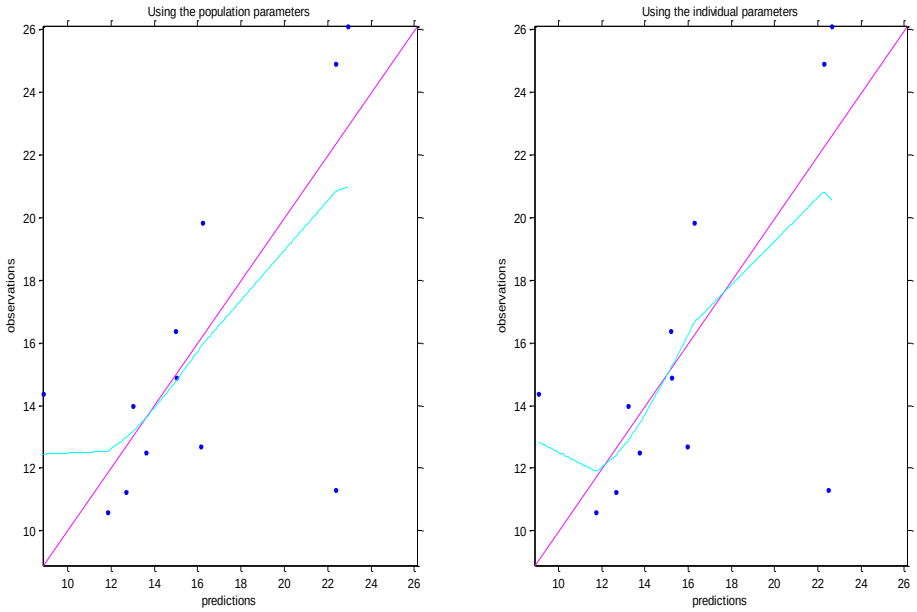


Figure 4.23 Goodness of fit plot for the observed and prediction concentrations in using population parameters (left) and using individual parameters (right) of group II and IV. Solid lines are the line of unity.

The model adequately characterized the observed concentrations (Figure 4.23). A same variability between pop predicted vs. observed plasma fentanyl concentrations Individual predicted concentration demonstrated distribution around the line of unity as same as pop prediction. The data point in this group had only 13 points, may not be clear for comparison.

The box plots of covariate and parameters showed in Figure 4.24 and 4.25. The graph showed the log volume of distribution of group II was more than group IV. But clearance of group IV was more than group II.

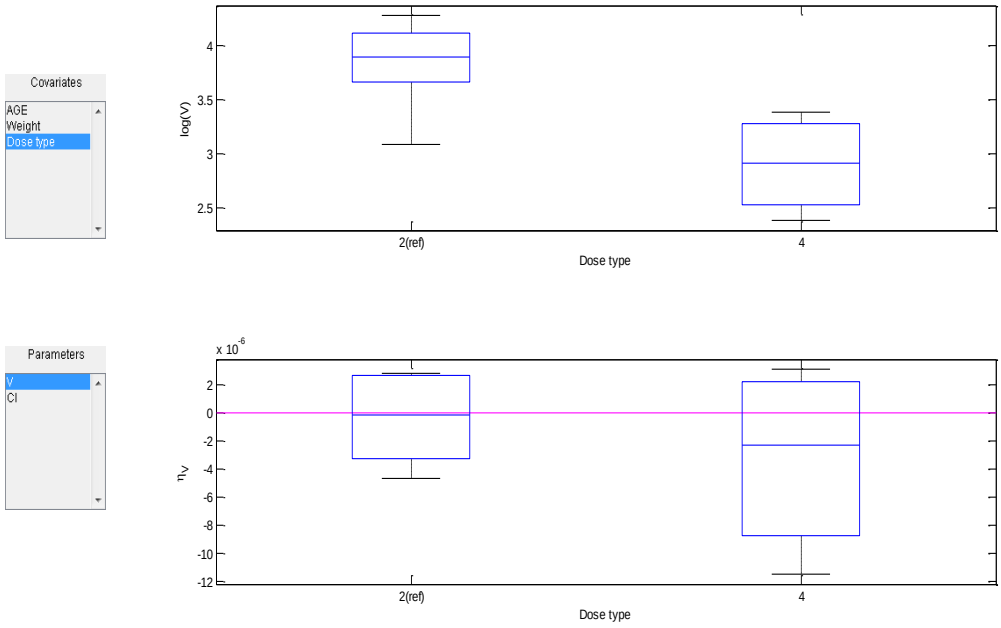


Figure 4.24 Box plot of Dose type and Volume of distribution. (group II and IV)

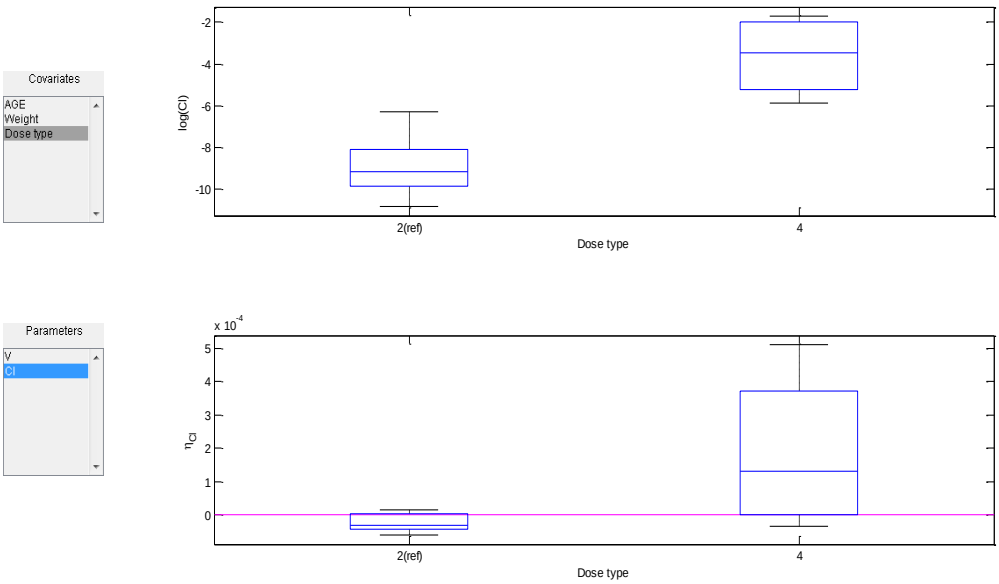


Figure 4.25 Box plot of Dose type and Clearance. (group II and IV)

Table 4.21 Estimation of the population parameters with low dose : (single-bolus and bolus-bolus group)

	parameter	s.e. (lin)	r.s.e (%)	p-value
V	0.0193	0.066	344	
beta_V (age)	0.0756	0.048	64	0.12
beta_V (weight)	0.0485	0.013	28	*0.00029*
beta_V (dose type_4)	-1.05	0.62	59	0.091
Cl	5.6e+009	6.5e+012	1.15e+005	
beta_Cl (age)	-0.331	14	4.3e+003	0.98
beta_Cl (weight)	-0.17	5.7	3.34e+003	0.98
beta_Cl (dose type_4)	5.44	20	368	0.79

Log-likelihood estimation by importance sampling

Sampling distribution for the random effects: t with 5 d.f.

-2 X log-likelihood: 67.96 (0.019)

Akaline Information Criteria (AIC): 89.96 (0.019)

Bayesian Information Criteria (BIC): 92.13 (0.019)

The parameters of this group were evaluated in population pharmacokinetic. If the parameters are significant at $p < 0.05$, covariate values were significant related. In group II and IV, only the volume of distribution was significant associated with body weight.

All of group, no matter group I and II, I and III, or II and IV were significant covariated log volume of distribution and body weight.

For high dosing groups : Group I, III and V (pooled data of high dosing groups) were estimated by Monolix® program by using initial value of single bolus high and low dose. Parameters would be compared by using one-way analysis of variance (1 way ANOVA). The level of significance was taken by p-value of 0.05. The analysis results are shown in Figure 4.26-4.30 and Table 4.22-4.23.

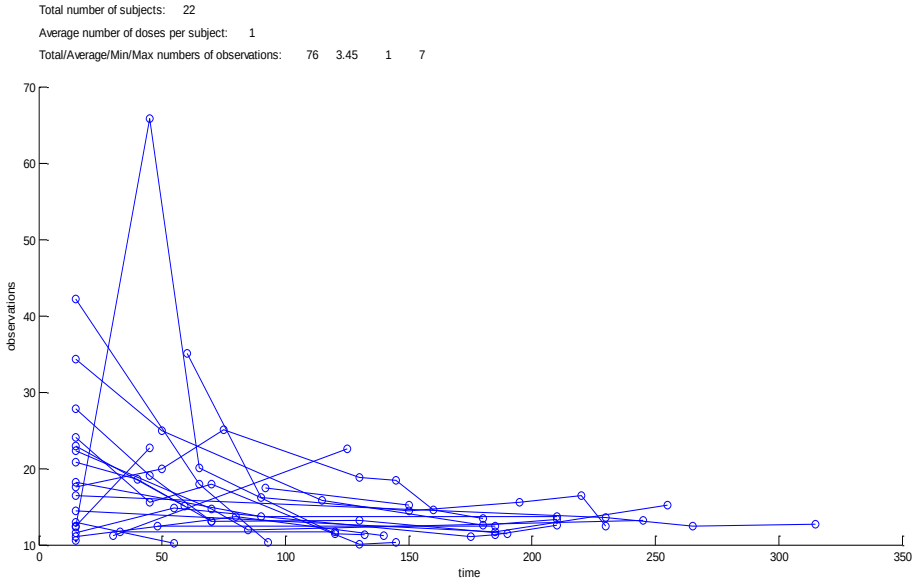


Figure 4.26 The spaghetti plot of group I, III and V. (pooled data of high dosing groups)

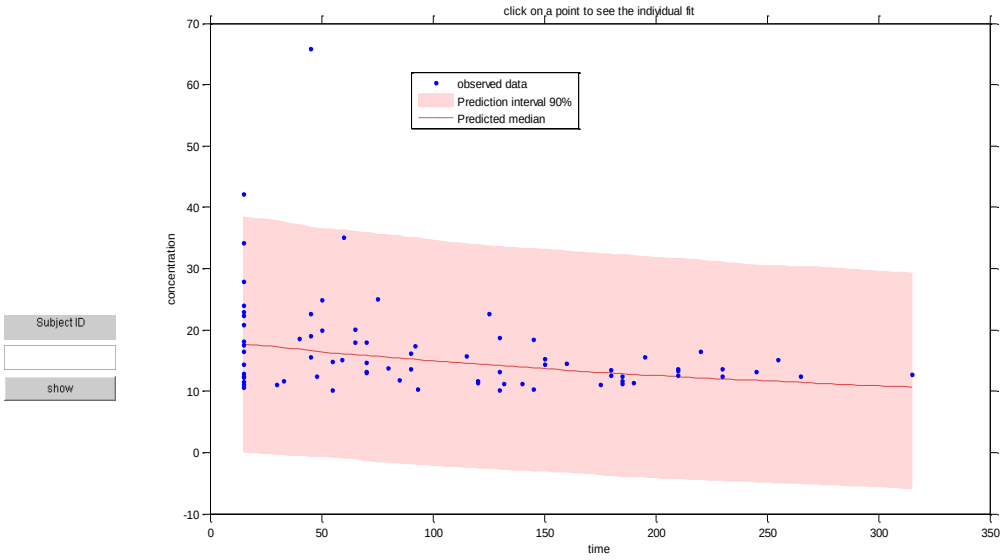


Figure 4.27 Observed data and prediction interval of group I, III and V. Graph shows mean with 90% prediction intervals of simulated concentrations in the pink interval. And the red line was the prediction median.

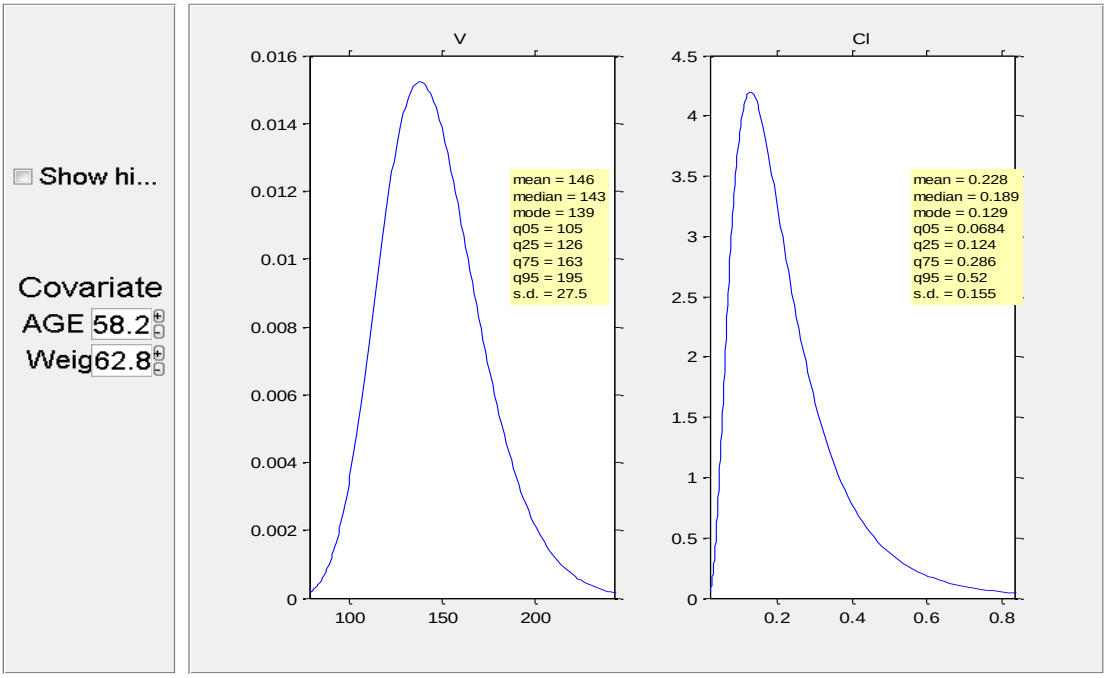


Figure 4.28 Volume of distribution and Clearance with mean age and mean weight covariate. (pooled data of high dosing groups). The data of parameters showed in Table 4.22.

Table 4.22 Volume of distribution and Clearance of high dosing groups. (pooled data)

Parameters	V_d (L) Mean ± S.D.	Cl (ml/min) Mean ± S.D.
Pooled data of high dose groups	146 ± 27.5	0.228 ± 0.155

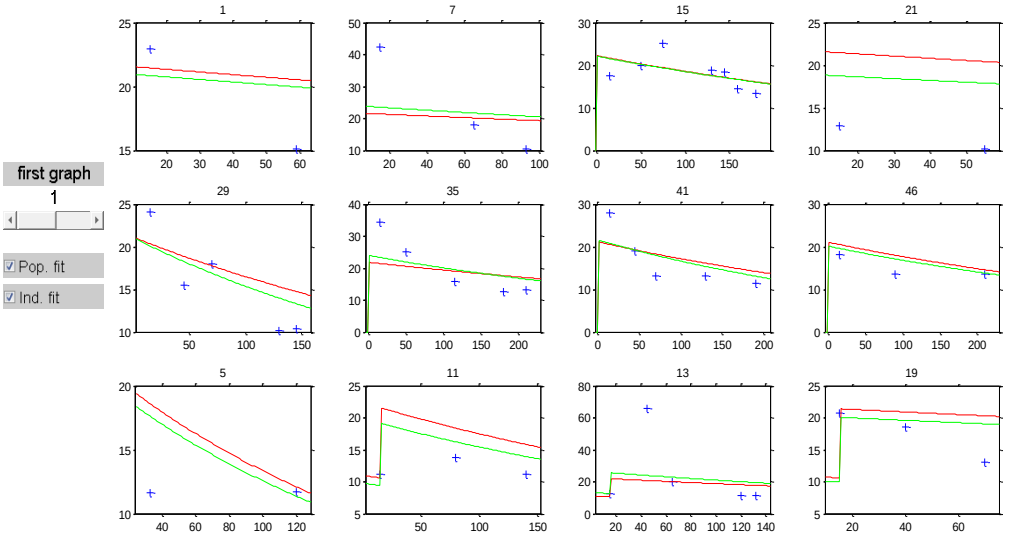


Figure 4.29 The individual fit that estimate by Monolix® program. (pooled data of high dosing groups) The green line represented the individual fit and the red line which represented the population fit.

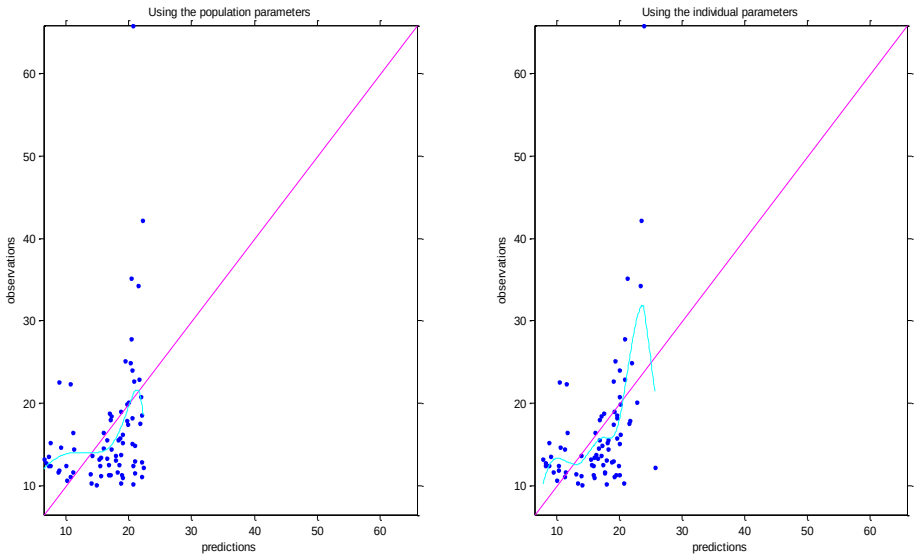


Figure 4.30 Goodness of fit plot for the observed and prediction concentrations in using population parameters (left) and using individual parameters (right) of group I, III and V.(pooled data of high dosing groups) Solid lines are the line of unity.

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Table 4.23 Estimation of the population parameters with high dosing group: (pooled data)

	parameter	s.e. (lin)	r.s.e (%)	p-value
V	47.7	53	110	
beta_V (age)	0.00128	0.012	972	0.92
beta_V (weight)	0.0163	0.0099	61	0.099
Cl	0.109	0.61	559	
beta_Cl (age)	-0.0361	0.063	175	0.57
beta_Cl (weight)	0.0422	0.049	116	0.39

Log-likelihood Estimation by linearization

-2 x log-likelihood: 535.84

Akaike Information Criteria (AIC): 553.84

Bayesian Information Criteria (BIC): 563.66

The parameters of high dosing group were evaluated in population pharmacokinetic. If the parameters are significant at $p < 0.05$, covariate values were significant related. The covariation of high dosing group (group I, III and V) did not significantly correlate between the body weight and pharmacokinetic parameters.

Two Couple of dosing group comparison: Group III and IV, Group V and VI which were the difference of bolus-bolus group and bolus infusion group, cannot run with Monolix® program because the variation of time of first injection and second injection / infusion time was very difference in individual patient. Moreover, an obstacle for operated duration in each person was more variation and less amounts of injection points in each group (BQL). The BQL values unable ran in any program of population pharmacokinetics.

CHAPTER V
DISCUSSION

Although fentanyl has been widely used in the operating theater and intensive care unit (ICU), its dosing strategy is unclear. This study provides pharmacokinetic explanation of fentanyl in cardio-pulmonary bypass graft (CABG) patients. The research is divided into two parts, 1) the method validation of an HPLC-UV VIS spectrophotometry and quantification of fentanyl in human plasma samples and 2) the population pharmacokinetic parameters with correlated results.

A HPLC method was developed and validated for quantification of fentanyl in human plasma. The HPLC-UV is the suitable and appropriate method for evaluation of fentanyl due to its high sensitivity, specificity, inexpensive sample preparation and ease of samples handling procedure in clinical practice. However, the major concern in the assay of this study was the interfering peak from midazolam (benzodiazepine for sedative effect) which was co-administer with fentanyl during the surgery. Moreover, fentanyl had UV absorbance at 210 nm which was near a limited cutoff of organic solvent (190 nm). Second, the method of validation of fentanyl using a cyano-HPLC column was not suitable for long period with acidic mobile phases. The cyano-HPLC column (CH_2-CH_2-CN) was tolerated in the narrow pH range (2-8). With decreasing the pH of mobile phase, the specificity and selectivity became poor (4). It should be note that the cyano-column was sensitive to low pH values. Third, we are aware of the fact that our HPLC assay of fentanyl is less sensitive than gas chromatography with mass spectrometry (GC-MS) or liquid chromatography with mass spectrometry (LC-MS). However, the cost of analysis of GC-MS or LC-MS is far more expensive than HPLC-UV method. The study describes the bioanalytical method for the quantification of fentanyl in human plasma. The expected concentration range of fentanyl in plasma was 15-60 ng/ml. The plasma concentration-time profile of fentanyl could be described its the pharmacokinetic patterns and sedative effect in CABG patients.

The method showed to be within an acceptable range for precision, accuracy and stability, therefore, were accepted for pharmacokinetic applications. The advantages of HPLC-UV in this study were 1) the reverse phase column (C-18) was tolerated with a high buffer concentration in low pH (pH 2.8), 2) not to change the column frequently, 3) used in long period of runtime (24 hours per batch) 4) the maintenance budgets is cheaper than GC-MS or LC-MS. Moreover, the validation design was created for a less spike of blood plasma which use only 300 µl and extracted by liquid-liquid extraction in small size of plastic tube (2.5 ml).

Although, there is HPLC assays for fentanyl measurements in many literature, we can found simultaneous fentanyl and midazolam concentration in the same system. Simultaneous of two drugs in the same system reduces costs and time. But in this study the midazolam peak was interfered by blank plasma. During the method development, the ratio between buffer and organic solvent as varied in order to achieve a good separation of fentanyl and midazolam. In this study, dimenhydrinate was used as internal standard. However, the retention time of dimenhydrinate was more than 22 min and the total time was 40 min per injection so it was the long period of time for running in each batch. The washing procedure must recommend after fentanyl quantification and change the guard column if necessary.

For the sampling design, an anesthesiologist collected blood samples 7-10 points all the surgery procedure. The period of procedure depended on the blood vessels for graft preparing, the number of how many bypass grafts and physiological responses in each patient. Plasma samples obtained from each patient may not be sampled at the same time, so there had variability of time samplings. One major concern in the assay was the limit of quantification of fentanyl in plasma. In high single-bolus and bolus-bolus dosing groups, the many data points were easier validated than the low dosing groups. The below quantitative level (BQL) points in low dosing group make the missing data and cannot predict in any pharmacokinetic model. The analytical method should be change to GC-MS or LC-MS, in order to obtain more accurately fentanyl concentration. The data points were very important for individual pharmacokinetic analysis. From the clinical data of fentanyl, it is a high volume of distribution and it has good distributed in fatty part of body. So, the study protocol may be increase number of sampling points in the initial procedure especially

in the first one hour of surgery procedure (from start to sedative induction to sternotomy period) or increase the number of subjects in each group of dosing type more than 8 persons which had men and women variable.

Because of the limited of data points, we decided to analyze the population pharmacokinetic by two programs: WinNonlin® and Monolix® program. WinNonlin® program was using sparse model (one-compartment model) to estimate initial values of volume of distribution (V_d) and clearance (Cl), followed by Monolix® program described population parameters and correlation results. The limited of sparse model in WinNonlin® required a rich data to find exactly initial parameters, for the more accurate estimation in the next step. About Monolix®, the one-compartment model with the inter-subject variability in log-normal model and the residual error in constant coefficient of variation model was selected as a base model. Quantitative covariates were evaluated with linear-normal distribution methods. P-value less than 0.05 was considered statistically significant. These programs were limited determination lower BQL in every time profiles in individual patient. The two or three compartment model cannot compare in this study. Moreover the correlation of sex category probably not determine because in the low single bolus group (group II) and low bolus-bolus group (group IV) had only men patients. So, the correlation of sex with other parameters could not study in all groups. The correlation of bolus-bolus group (group III and IV) and bolus-infusion group (group V and VI) were not estimated because the many BQL levels which were not enough data for evaluation by Monolix® program. The covariate correlations with pharmacokinetic parameters were age, body weight, and dosage regimens.

Population pharmacokinetic parameters were significant difference between high- and low-dosing groups. Assume for this experiment that the variance of volume of distribution (V_d) depends on the log of body weight. In the different of couple groups: single-bolus group and bolus-bolus group in high dose (group I and III) were significant difference of volume and clearance in difference dose type but not different in low dosing group (group II and IV). About the high and low single bolus dose (group I and II), low dosing type was significantly difference the volume of distribution from high dose. It means the amount of dosing had effect for pharmacokinetic parameters. All of the volume of distribution (V_d) results had

straightforward determination between fenatnyl level and log of body weight. It is in agreement of pharmacokinetic data of fentanyl that it is binding with lipid tissues and much volume of distribution in body. That's a pitiful results, bolus-infusion group was not explained in any program.

The pooled data of high dosing groups (group I, III and V) which the group was predicted the population pharmacokinetic result. The volume of distribution and clearance were 47.7 liters and 0.109 ml/min, respectively. These values can be used in as basic information for further study in related fields. Moreover, the body weight was not significantly correlated with any pharmacokinetic parameters as same as sub-divided groups. It could be refer the pharmacokinetic parameters of fentanyl independent the age and body weight factors.

This study, although conducted under restricted intraoperative circumstances, attempted to offer the pharmacokinetic explanation has been no clear individualized dosing strategy. In combination with pharmacodynamic data, accurate and precise pharmacokinetic models such as the one we had defined, provided the scientific foundation for designing dose regimens that could be achieve these goals in a reliable and predictable manner. About the compartment of analysis is a program's limitation, the burn patient in previous study used the three-compartment of analysis. But in this study two or three compartment cannot use anymore. All of data will be estimate by one-compartment model only.

The current study has a few limitations. First, the LLOQ of the developed method was 10 ng/ml. The blood samples were not collected at the same time points in each patient. The lack of vital pharmacodynamic data of each patient in chronological order. Second, some of the well-recognized patient-specific variables on fentanyl pharmacokinetics were not recognized as important covariates in the modeling process. We recruited a relatively homogenous population to emphasize the decisive difference between dose regimen and pharmacokinetic results. A dataset was obtained from a small of number of subjects in each group. Thus variables such as age, remained with a narrow range, compromising their significance in modeling. Age-related pharmacokinetic alterations have been demonstrated for fentanyl (54). The relationship between weight and fentanyl requirements is linear below 100 kg body weight (55). Decrease plasma protein binding in renal dysfunction may potentially

alter the free fraction of the fentanyl (56). Reduced liver blood flow and metabolic function due to liver disease would delay the decline of fentanyl plasma concentration (57). Although the control was included in inclusion criteria before selected the patient into this study, the relative factors in operating room had effect for fentanyl pharmacokinetic results. Cardiac output and heart-lung machine was not identified as a significant covariate in our analysis. It has been recognized as an important factor affecting fentanyl pharmacokinetics in the experimental setting. The difference in simulation of plasma drug concentration time profile could only in part of explanation. Although the pharmacokinetics parameters were largely alters, the difference in simulation of plasma drug concentration-time profiles could only part explain tremendously augmented resistance to analgesic in the CABG patients, roiling out altered pharmacokinetics as the primary cause.

In the future studies, the model for designing dose regimens of fentanyl should be developed. In combination with intra-operative fentanyl pharmacodynamic data, anesthesiologists have a rational foundation for designing fentanyl dosage regimens that could maximize the benefits of opioids during surgery. The method of quantification fentanyl in blood plasma, to prove this in a more specific way, NONMEM and simulation were utilized to analyze and present data.

CHAPTER VI
CONCLUSION

A reversed-phase high performance liquid chromatography method for the determination of plasma concentration of the narcotic analgesics, fentanyl using dimenhydrinate as the internal standard is presented. Chromatographic separations were achieved with a reverse-phase C18-HPLC column. The assay was linear over the expected concentration of fentanyl in the CABG patients. The results obtained for intra-assay and inter-assay precision and accuracy satisfactorily met the internationally established acceptance criteria for bioanalysis. The assay procedure was applied in a human of pharmacokinetic studies of fentanyl during cardiac surgery. This research provides a better elucidation of pharmacokinetic variables in this populace. We are aware of the fact that our assay for fentanyl was less sensitive than mass spectrometry-based analytical methods such as gas or liquid chromatography-mass spectrometry (GC-MS or LC-MS). However, the cost of analysis of this method is far more expensive than an HPLC-UV method.

Our pharmacokinetic model provides a rational foundation for designing fentanyl dosage regimens for patients undergoing cardio-pulmonary bypass graft (CABG). Forty-eight adults, age 52.8 years, with 68.2 (mean) body weight, were enrolled at 6 groups. Each patient with inclusion criteria was received fentanyl in a low or high dosing in single bolus, bolus-bolus and bolus-infusion regimens. After an intravenous of fentanyl, the plasma concentrations were sequentially determined up to finished surgery procedure. Concentration-time profiles were subjected to sparse-model with WinNonlin® for evaluation initial parameter values of volume of distribution (V_d) and clearance (Cl) and correlation parameter with covariate population pharmacokinetic results by Monolix® program.

The pharmacokinetic results showed in every group, the volume of distribution had correlated with body weight. An each couple group estimation, low and high dosing types were different results but had only correlation between volume

of distribution and the log of body weight. In contrast, the population pharmacokinetic parameters of pooled data of high dosing groups did not correlate with any covariate factors. The best regimen may not be concluding in this time because the pharmacodynamic data had not evaluated in this research. The fentanyl concentration-time profile was directly calculated in one-compartment population pharmacokinetic which lacked of other compartments evaluation because the below quantification levels in some groups.

This study, although conducted under restricted intra-operative circumstances, attempted to offer the pharmacokinetic explanation which has been no clear individualized dosing strategy. The design of number of patient in each dosage regimens should be different in sex variability, time samplings in initial surgery procedures may be frequently collected, the method for quantification of fentanyl concentration should be cover in BQL ranges. The effect of BQLs to the analysis is a program's limitation, the two or three compartment cannot use for analysis. Although pharmacokinetic parameters were only explained in this study, our data would offer better understanding of fentanyl in population and had benefit for future design.

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