DEVELOPMENT OF LIQUID CHROMATOGRAPHY MASS SPECTROMETRY METHODS FOR THE MONITORING OF ROPIVACAINE, ENDOGENOUS STEROIDS AND DIHYDROARTEMISININ

A thesis submitted for the degree of Doctor of Philosophy at the University of Strathclyde

By

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Declaration

‘This thesis is the result of the author’s original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.’

Signed:

Date:

Acknowledgement

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List of abbreviations and acronyms

µg:	Microgram
µL:	Microlitre
17β-E_2:	Estradiol
ACN:	Acetonitrile
AGP:	α_1-Acid glycoprotein
APCI:	Atmospheric pressure chemical Ionization
ATP:	Adenosine triphosphate
BBB:	Blood brain barrier
Cmax:	Maximum concentration of the drug in plasma
Cl:	Clearance
CNS:	Central nervous system
CYP450:	Cytochrome P450 enzyme
DHA:	Dihydroartemisinin
E_1:	Estrone
EC_{50}:	Effective concentration in 50% patients
ED_{50}:	Effective dose in 50% patients
EI:	Electron impact ionization
ER_α:	Alpha estrogen receptors
ER_β:	Beta estrogen receptors
ESI:	Electrospray ionization
eV:	Electron Volt
FDA:	Food and drug adminstration

FDMPTS:	2-Flouro-1,3-dimethyl p-toluene sulfonate
FDT:	Free drug theory
FTMS:	Fourier transform mass spectrometry
fu:	Fraction unbound in plasma
fuT:	Fraction unbound in tissue
g:	Gram
HDL:	High density lipoprotein
HILIC:	Hydrophilic interaction liquid chromatography
HPLC:	High performance liquid chromatography
IVRA:	Intravenous regional anaesthesia
kV:	Kilovolt
LC-MS:	Liquid chromatography mass spectrometry
LDL:	Low density lipoprotein
LH:	Luteinizing hormone
LIA:	Local infiltration analgesia
LLE:	Liquid-liquid extraction
m/z:	Mass/charge ratio
MALDI:	Matrix assisted laser desorption ionization
mg:	Milligram
MLAC:	Microextraction in packed syringe
MLAC:	Minimum local analgesic concentration
mM:	Millimolar
ng:	Nanogram

ODS:	Octadecyl silane
pg:	Picogram
PPT:	Protein precipitation
RED:	Red equilibrium dialysis
RPC:	Reverse phase chromatography
rpm:	Revolutions per minute
SPE:	Solid phase extraction
Tmax:	Time to reach maximum concentration in the plasma
TFA:	Trifluoro acetic acid
THA:	Total hip arthroplasty
TKA:	Total knee arthroplasty
TOF:	Time of Flight
v/v:	volume/volume
V_d:	Volume of distribution
VLDL:	Very low density lipoprotein
V_p:	Plasma volume
V_t:	Tissue volume
w/v:	weight/volume

Previously published work

1. Zhang T, Watson DG, Wang L, Abbas M, Murdoch L, et al. (2013) Application of holistic liquid chromatography-high resolution mass spectrometry based urinary metabolomics for prostate cancer detection and biomarker discovery. PloS one 8: e65880.
2. Abbas, M., L. Ahmad, et al. (2013). "Development of a method to measure free and bound ropivacaine in human plasma using equilibrium dialysis and hydrophilic interaction chromatography coupled to high resolution mass spectrometry." Talanta 117: 60-63.
3. Gill, A., N. Scott, et al. (2014). "Ropivacaine plasma levels following local infiltration analgesia for primary total hip arthroplasty." Anaesthesia 69(4): 368-373.
4. Brydone, A., R. Souvatzoglou, et al. (2015). "Ropivacaine plasma levels following high-dose local infiltration analgesia for total knee arthroplasty." Anaesthesia.

Abstract

The thesis consists of five chapters.

The first chapter is general introduction to LC-MS, separation technology and extraction methods.

The second chapter describes the development of a method to measure free and bound ropivacaine in human plasma using equilibrium dialysis and HILIC chromatography coupled with a high resolution mass spectrometery. In this chapter a highly sensitive method for the determination of the free and bound portion of ropivacaine in patients undergoing knee and hip joint surgery was developed. Patient samples were provided by the Golden Jubilee Hospital, Clydebank. The method was validated according to FDA guidelines.

The third chapter describes the determination of α1-glycoprotein in the plasma of patients undergoing knee and hip joint surgeries using an already established method using HPLC with a polymeric reversed phase column, thus enabling comparison of the concentration of AGP protein with the levels of bound and unbound ropivacaine. This complete set of data should allow pharmacokinetic modelling studies to be carried out.

The fourth chapter covers the determination of the steroids estradiol and estrone and their hydroxyl and methoxy metabolites following a derivatization reaction for provision of improved sensitivity. The free steroids are not readily ionized by ESI mass

spectrometry and the derivatization procedure produces positively charged ions in order to enhance the detection of these steroids. Different reagents were used and the best sensitivity was obtained by using 2-fluoro 1, 3-dimethylpyridinium *p*-toluene sulphonate as the derivatising agent. This method was applied to samples obtained from patients with pulmonary hypertension in order to evaluate the role of steroids in the progression of disease.

The fifth and last chapter covers the determination of an anti-malarial drug artemether and its active metabolite DHA in human plasma using a reversed phase C18 column coupled with high resolution mass spectrometry, the samples were provided by the University of Peshawar, Pakistan.

Chapter 1

1 General introduction

1.1 Liquid chromatography mass spectrometry

Liquid chromatography is used for the physical separation of components of a mixture using two phases, a mobile phase and a stationary phase. This method is most commonly used for the quantitation of drugs in formulations. Liquid chromatography nowadays is often used in combination with mass spectrometry systems. LC-MS is considered to be a very powerful analytical technique in drug research analysis particularly for the separation and identification of drug metabolites. The components of a mass spectrometry system, the columns used in liquid chromatography for separation of various compounds and different extraction procedures used in the preparation of samples for analysis are discussed below [1].

1.1.1 Mass spectrometry systems

Mass spectrometry is an analytical tool which is used for the analysis of small organic molecules as well as large macromolecules. It works on the basis of formation of gas phase ions (either positively or negatively charged) which are then separated on the basis of their mass to charge ratio (m/z). So it is necessary for the substance to evaporate or be volatilized in the mass spectrometry system [2].

All mass spectrometers generally share the same parts which include: an ionization source, mass analyser and a detector as shown in figure 1.1 below.

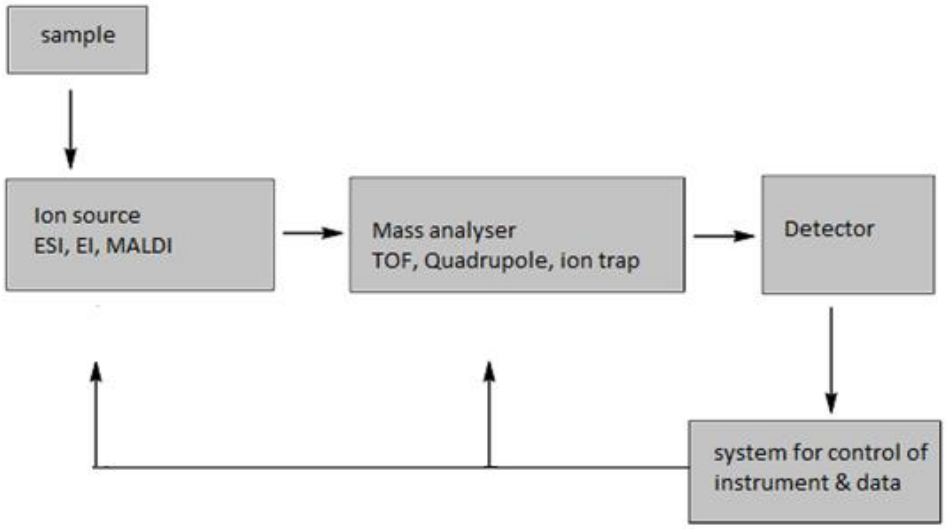


Figure 1.1 Basic components and general representation of a mass spectrometry system

1.1.1.1 Ionization techniques

Various ionization techniques can be used in mass spectrometry for the formation of gas phase ions and these are described below.

1.1.1.1.1 Electrospray ionization (ESI)

ESI has been the most widely used ionization technique for the last two decades because of its compatibility with HPLC. In ESI the analytical solution is pumped through a needle at high potential. A very high voltage normally 3 to 4 kv is applied to the tip of the needle, which is present in the ionization source of the mass spectrometer (figure

1.2). If a positive potential is applied at the tip of the needle then the negative ions in the effluent are attracted towards the needle and when a negative potential is applied then positive ions are attracted. As a result of this strong electric field the sample solution emerging from the tip of the needle is dispersed into a very fine spray of highly charged droplets due to coulombic repulsion, this process is also supported by a coaxial flow of nebulising gas (nitrogen) that flows around the outside of capillary. The drops under the repulsive forces produced by the accumulated ions elongate and form a Taylor cone which finally splits down to smaller droplets which are further reduced in size by the evaporation of solvent with the help of a warm flow of drying gas passing across the front of ionization source. The drying gas is normally nitrogen. Finally the charged sample molecules without solvent are released from droplets as a result of an internal replusive force and the ions are then transferred to the high vacuum region of mass analyzer. ESI is considered to have good sensitivity but it is also sensitive to matrix effects which may cause signal suppression [3].

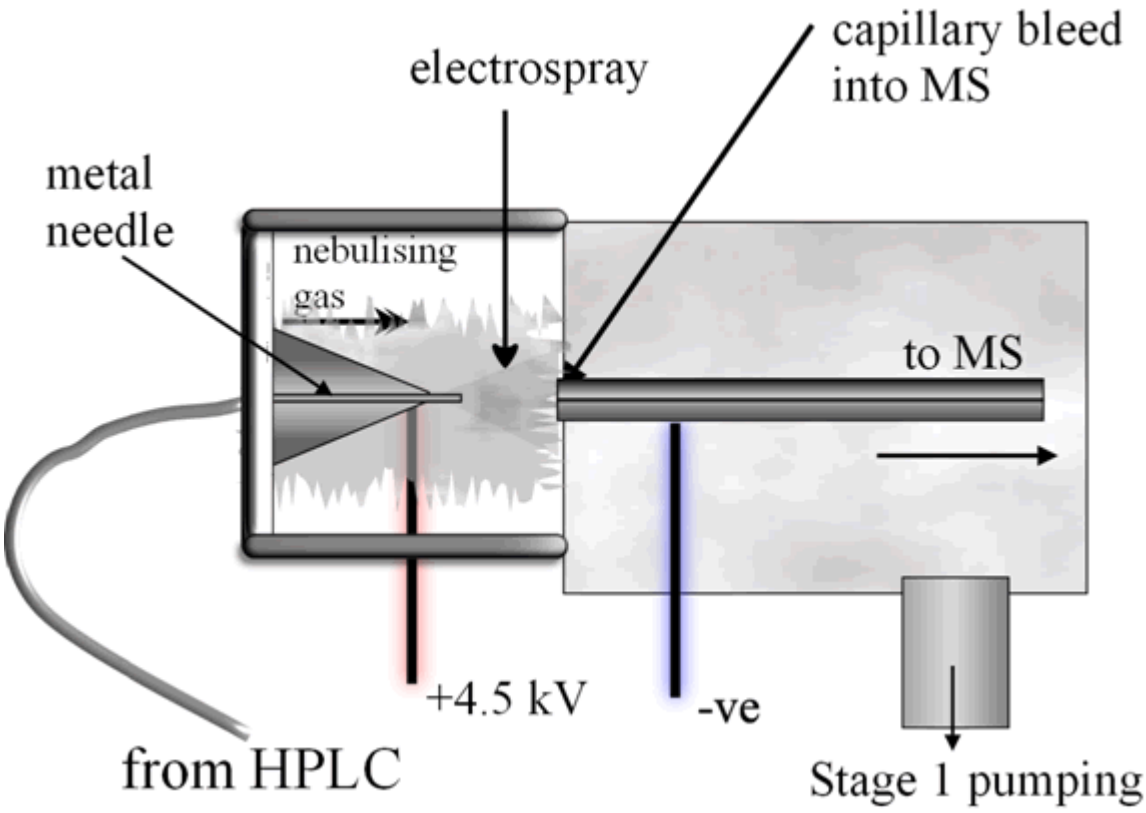


Figure 1.2 The electrospray ionization process showing the formation of ions in the mass spectrometer

1.1.1.1.2 Atmospheric pressure chemical ionization (APCI)

Atmospheric pressure chemical ionization is considered to be very close to ESI mode in its operation and the two ionization techniques can be used by switching one mode to another in the same instrument. In APCI the eluent coming from the HPLC passes through a heated tube instead of passing through a charged needle and forms an aerosol. An electric discharge is then passed through the aerosol as it exits from the heated tube which results in the formation of highly reactive species like H_3O^+ and N_2^+ which then cause the ionization of the target compounds. This technique is not as popular as ESI and is used for the ionization of compounds of low polarity [4].

1.1.1.1.3 Electron impact ionization (EI)

Electron impact ionization is the oldest ionization method however this method is not compatible with HPLC as tool of introducing the analyte into the mass system. EI can be used in combination with the sample introduced into the mass system either by a direct heated probe or by gas chromatography. So the sample is introduced into the instrument by heating on the probe end until it evaporates with the help of high vacuum or alternatively by using a GC capillary column. After converting into the vapour phase a beam of electrons is directed toward the gas phase sample which are speeded up towards a positive target having a high energy of 70 eV (figure 1.3). When electron collides with the neutral molecule it ejects an electron from the molecule resulting in positively charged molecular ion.

$$M + e^- \rightarrow M^{+\cdot} + 2e^-$$

This technique is used only to produce positive ions. EI results in extensive fragmentation of an analyte as the electrons have much higher energy than the bond energies of the bonds within the analyte which are 4-7eV and the fragments produced are helpful in structure elucidation [5]. These fragments are then ejected from the source towards the ion separation device with the help of a repeller plate that has the same charge as the ions generated [6].

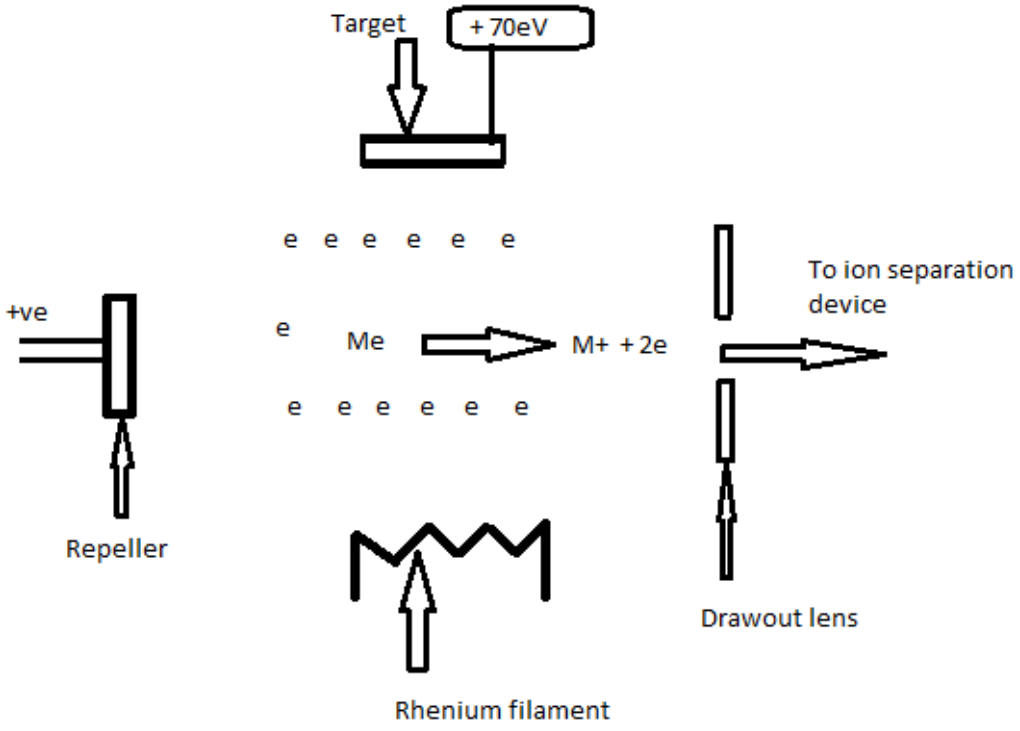


Figure 1.3 Mechanism of electron impact ionization, the diagram is redrawn from [6]

1.1.1.1.4 Matrix assisted laser desorption ionization (MALDI)

In MALDI the sample is first mixed with a suitable matrix and then ionization is carried out with the help of nitrogen laser. A matrix should absorb UV radiation produced by the laser around the wavelength of 337nm. An example of a matrix is dihydroxybenzoic acid. The sample is first mixed with the matrix usually on a metal plate and allowed to dry before introducing it into the instrument where a nitrogen laser is used for the ionization of the target analyte (figure 1.4). MALDI is generally used in combination with time of flight (TOF) separation for the analysis of high molecular weight

compounds such as proteins because it produces singly charged ions of proteins which can then be separated by time of flight (TOF). This technique like ESI is a soft ionization producing positive ions and negative ions without any extensive fragmentation [5].

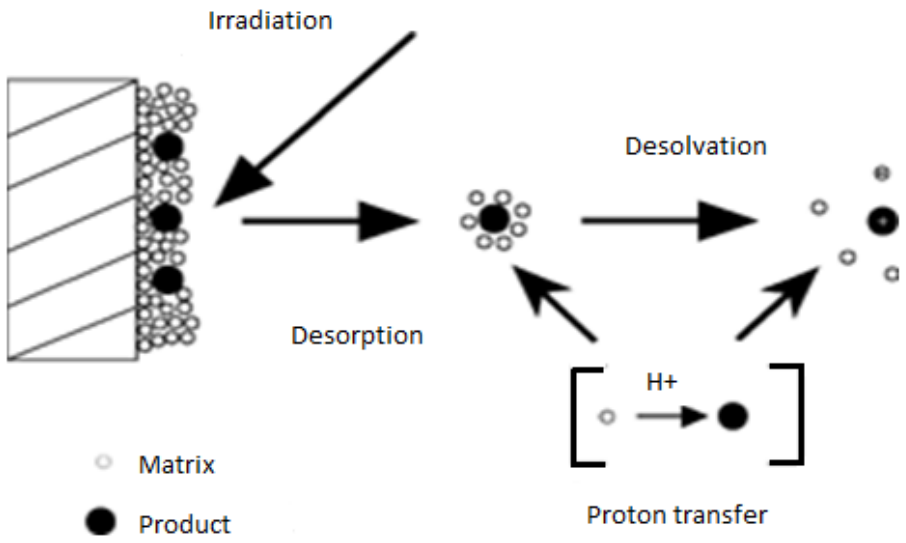


Figure 1.4 Matrix assisted laser desorption ionization showing the mechanism of ion formation, the diagram is reprodruce from [5]

1.1.2 Mass analyzers

1.1.2.1 Quadrupole analyser

Quadrupole analysers are relatively cheap and are the most commonly used analysers in bioanalytical methods based on tandem mass spectrometry. Quadrupole analyzers consist of four parallel rods which are connected in pairs having the same potential but with opposite signs (figure 1.5). In quadrupole analyzers two electric fields are applied

which are at right angles to each other for the separation of ions. These two fields are a DC field and an alternating radiofrequency (RF) field. The purpose of applying two electrostatic fields at right angles to each other is to produce a resonance frequency for each m/z value. Thus the ions which have the same resonating frequency as of the quadrupole are able to pass through and to be detected. Ions having m/z lower or higher than the resonant frequency at a particular instant will collide with the rods and will not reach the detector [7].

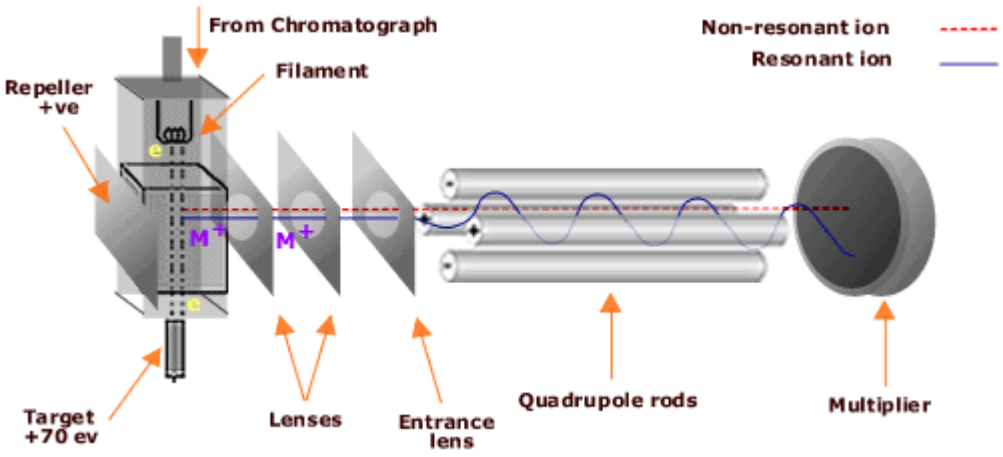


Figure 1.5 Separation of ions using quadrupole. Ions resonating with the frequency of quadrupole will pass through and detected by multiplier while ions having different frequency from quadrupole will strike the rods and will not reach the detector [7].

1.1.2.2 Time of flight (TOF)

Time of flight analyzers became commercially available around 1990. This technique is commonly used for the analysis of high molecular weight compounds like proteins. The technique is based on the principal that the smaller ions move more quickly in a long drift tube as compared to larger ions after accelerating them through an electric field

and are thus detected first by the detector. The time period during which the ions are ejected from the ion source is very critical and needs to be well defined in order for the technique to be effective. ESI can be used in combination with a gating mechanism which has the ability to allow the ion to access to the field of separation for only a short time period. The kinetic energies of the ions leaving the ion source are different which results in poor mass resolution so to avoid this problem another device called reflectron was introduced into the system to focus the kinetic energies of the ions. The ions having greater kinetic energy will penetrate further into the reflectron and as result the faster ions are retained by the reflectron allowing the slower moving ions to catch up [8].

1.1.2.3 Ion trap

Ion trap technology was first introduced commercially in the late 1980s. The analyte is first ionised with the help of one of the available methods and the ions are then introduced into the trap. The ions are then trapped by an Rf voltage which is applied to a circular electrode. The energy of the ions in the trap is quenched by helium which is introduced into the trap. The ions can then be ejected selectively from the trap on the basis of their masses with the help of a DC electric field applied to the endcap electrode (figure 1.6). If the fragmentation of a particular ion is desirable then the trap can eject all the ions except the ion of interest. The Rf voltage can then be changed accordingly to excite the ion which then collides with the helium in the trap to produce fragments which are then ejected and detected by detector [9].

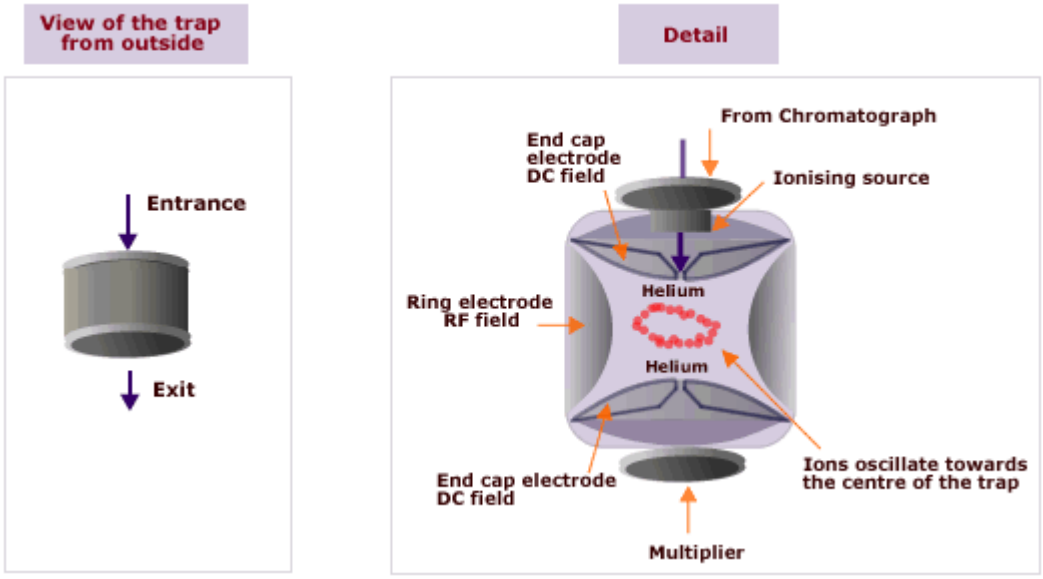


Figure 1.6 Schematic diagram of ion trap showing different parts of ion trap [9]

1.1.2.4 Fourier transform mass spectrometry (FTMS)

Fourier transform mass spectrometers are able to measure very accurately the mass of the target analyte. The masses are measured by the trapping the ions in a strong magnetic field. Orbitrap was introduced in 2005 is the most commonly used instrument which uses Fourier transform. In an Orbitrap (figure 1.7) an electrostatic field is used for trapping the ions rather than a magnetic field which is used in an ion cyclotron resonance instrument. The ions are trapped so that they move around a central spindle shaped electrode while oscillating in the Z direction. The oscillation of the ions is then detected in the form of an image current which is ultimately changed into very accurate mass information following Fourier transformation. The Orbitrap has the ability to measure mass to up to five decimal places [10].

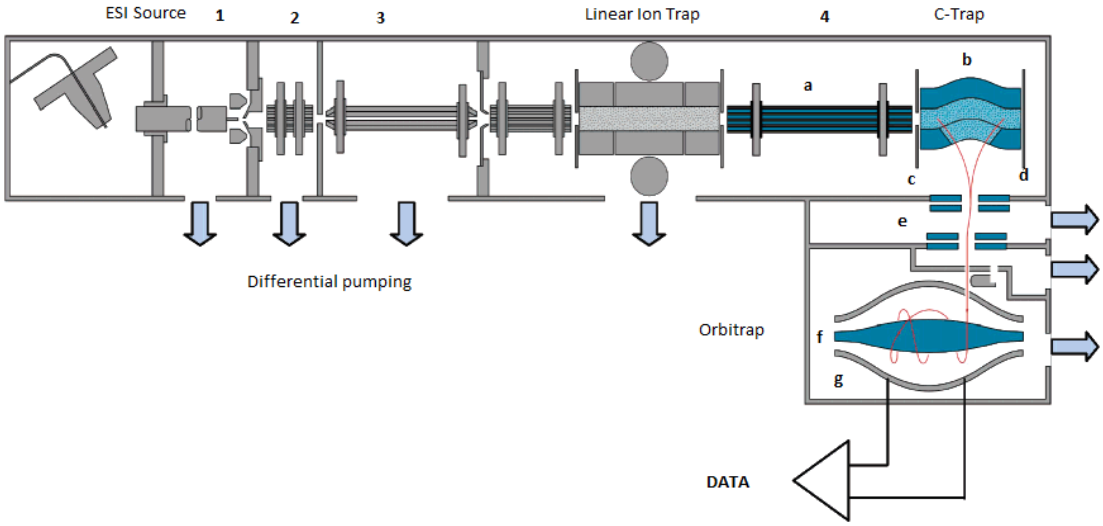


Figure 1.7 The schmetaic diagram of LTQ orbitrap showing it different parts i.e ESI source, linear ion trap, C-trap and orbitrap. The diagram is reproduced from [10].

1.2 Separation techniques

Chromatography techniques play an important role in the analysis of target analytes in various biological samples. Chromatography is a physical technique for separation of an analyte where the target analyte distributes between two different phases i.e. the stationary phase and the mobile phase. The physical nature of the stationary phase or chemistry of the column determines the selectivity of the method.

1.2.1 Reversed phase chromatography (RPC)

ODS silica gel is the most common material used for the packing of reversed phase columns. The separation mechanism in RPC is through the retardation of a compound by its partitioning into the stationary phase according to its lipophilicity. Apart from the stationary phase, the mobile phase also needs to be considered with regard to the

retention of a particular compound. In case of a RP column the more lipophilic a mobile phase the more quickly a compound elutes from the reversed phase column. In gradient RPC the mobile phase starts with high water content and ends with high organic solvent. These columns are mainly used for the analysis and retention of non-polar compounds [11].

1.2.2 Hydrophilic interaction liquid chromatography (HILIC)

HILIC is an alternative technique to RPC because polar compounds are not retained by reversed phase columns. This technique was first reported in 1990 by Alpert. HILIC columns are often silica based having a hydrophilic surface, among these ZIC-HILIC columns are very commonly used. In ZIC-HILIC columns a sulfobetaine zwitterionic functional group has been used to coat the silica thus allowing polar analytes to retard by partitioning in a hydrophilic environment. Apart from partitioning of analyte between the mobile phase and water enriched layer on the stationary phase, there is also a possibility of ion exchange interactions in case of strong acids and bases. In this type of chromatography the water at the surface of the stationary phase is behaving like a pseudostationary phase and separation works in a completely opposite manner to reverse phase chromatography. In this mode of chromatography the more polar the compound the more strongly it is retained on the column or the higher content of water in the mobile phase the more quickly it will elute the analyte [12].

1.3 Extraction methods for biological samples

1.3.1 Protein precipitation (PPT)

Protein precipitation is considered as the simplest and quickest method of sample pretreatment. In this method a solution or solvent is added to the tissue sample in order to denature the proteins and results in precipitation of proteins. There are a number of solvents used to precipitate the proteins but results show that deproteinization caused by acetonitrile results in efficient removal of protein and good recovery. Strong acids can be used for the precipitation of proteins but may lead to hydrolysis of some conjugates like sulphates and glucuronides. PPT is a rapid and cheap method used for the efficient removal of proteins and works for a wide range of compounds [13].

1.3.2 Solid phase extraction (SPE)

SPE is a technique used for the sample preparation and employs a chromatographic packing material in a cartridge to separate different components of a sample (figure 1.8). Its mechanism is very much similar to that of HPLC. SPE is very effective sample pretreatment technique for eliminating interfering substances thus enhancing sample purity and is frequently used for the sample pretreatment procedure. A wide variety of sorbents is available for SPE which includes reversed phase materials, both strong and weak ion exchange materials and mixed mode materials [14].

SPE is used for the extraction and purification purposes because of its high selectivity, its speed of extraction, automation potential and the quantity of organic solvent used in SPE is much less than used in liquid liquid extraction (LLE). SPE is thought to decrease the serum background to a greater extent as compared to the PPT method. Reversed phase SPE is widely used in biological analysis and is considered a versatile technique because it employs a wide range of cartridges and solvents. The effectiveness of the SPE depends on the nature of the sorbents, volume of the sample and its pH as well as volume of the solvent used for the elution [13].

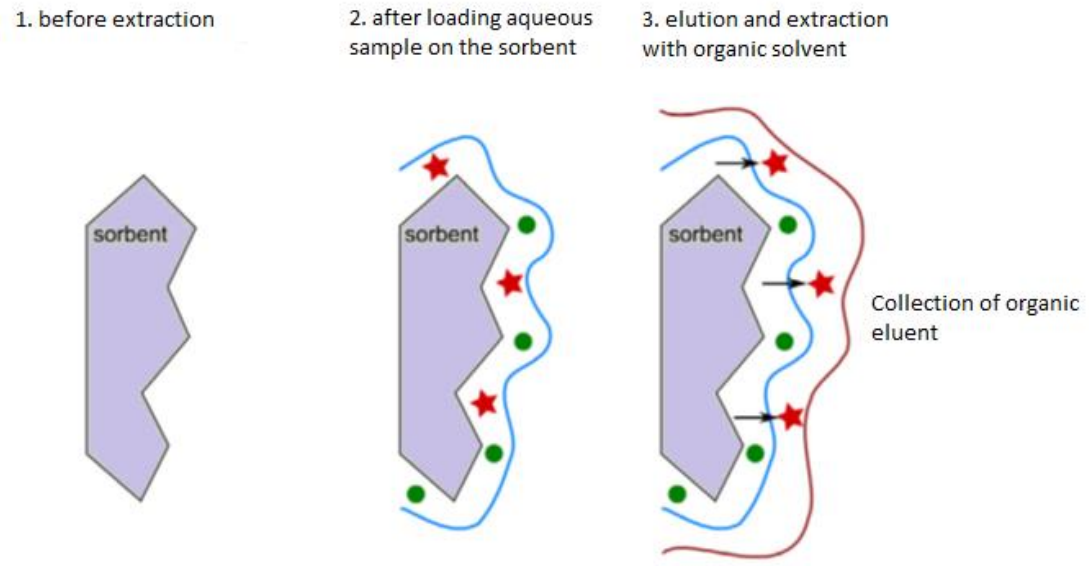


Figure 1.8 Mechanism of solid phase extraction, the stars in the figure represents analyte of interest while the green dots are matrix components, the blue line represent loading of the sample on the column while red line means collection of targeted analyte in organic solvent [15]

1.3.3 Liquid-liquid extraction (LLE)

LLE involves mixing of two immiscible solvents one of them is aqueous while the other one is organic and time is allowed for the two immiscible solvents to interact with each

other so that the organic layer extracts the analyte from the aqueous phase. There are a number of factors that have an effect on the selectivity and recovery of the target analyte from the aqueous phase. These factors include the solubility of the analyte and pKa value, pH of the solution and ionic strength. Once the two immiscible solvents are separated by centrifugation, the organic layer having the analyte of interest is then removed, evaporated to dryness and then reconstituted in a suitable organic solvent for LC-MS analysis. The main advantage of this method is that the careful selection of solvent and pH results in very clean extracts having a very good selectivity for analyte of interest but the disadvantage is that is more time consuming than other methods [16].

Chapter 2

2 Determination of Free and Bound Ropivacaine in Human Plasma Using Equilibrium Dialysis and Hydrophilic Interaction Chromatography Coupled to High Resolution Mass Spectrometry

2.1 Local anaesthetics

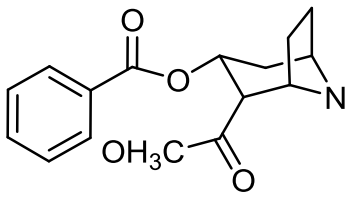
Local anaesthetics act by reversibly inhibiting the conduction of sensory nerve impulses regionally and are thus very useful in peri- and post-operative relief of pain without having any effect on consciousness [17]. Also the reversible nerve impulse inhibition caused by long acting local anaesthetics is helpful in prolonging sensory and motor nerve blockade and thus can be used to produce anaesthesia in various kinds of surgeries. The sensory blockade which is produced at low doses is suitable for labour pain, postoperative pain and other types of acute pain relief but the accompanying blockade of motor nerves has no benefits and is generally not desirable [18].

Bupivacaine, which is an amide local anaesthetic from the pipecoloxylidide group of compounds, has been widely used for many years as a long acting anaesthetic for inducing local anaesthesia, but in 1979 Albright [19] diverted the attention of anesthesiologists to the serious matter of a cardiac arrest that might be related to the intravascular administration of bupivacaine. Also CNS and cardiovascular toxicity has

subsequently been reported which are thought to be linked with the drug's *R*-enantiomer after excessive administration of bupivacaine [17].

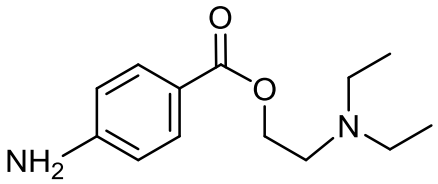
2.1.1 Historical background

In 1860 Albert Niemann, a German chemist isolated cocaine from the leaf of coca and later on in 1884 it was used for the very first time clinically as a local anaesthetic by Carl Koller [20] who was Viennese ophthalmologist when he performed the first surgery of a glaucoma patient. However, the initial extensive use of cocaine as a local anaesthetic was curtailed because there were about 200 cases regarding its systemic toxicity and 13 deaths reported in relation the use of the drug during 1884 to 1891. So the search started again for a safer therapeutic alternative to cocaine.



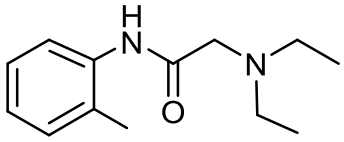
Strcuture of Cocaine

In 1904, Alfred Einhorn, a German chemist synthesized a new compound called novocaine which was renamed procaine later on in United States. Based on its safety profile, it became the first choice as a local anaesthetic until some allergic reactions were found to be caused by this drug in patients.



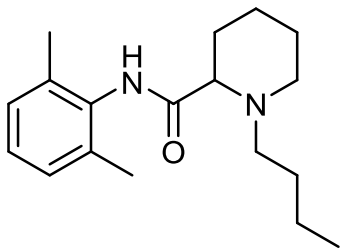
Structure of Procaine

In 1943 two scientists Lofgren and Lundquist developed the first ever local anaesthetic with an amino-amide group, it was a xylidine derivative and was named lidocaine. Lidocaine came to the market in 1948 for the first time and has been used clinically for over 60 years. Lidocaine was considered to be one of the safest and most efficacious local anaesthetics ever produced and was used worldwide for its safety and efficacy, although it has a short duration of action which is its main disadvantage [21].



Structure of Lidocaine

In 1957 Boaf Ekenstam [22] synthesized bupivacaine and after ten years he introduced it into clinical practice. Bupivacaine is a local anaesthetic of amino amide group class and is considered as a long acting agent. It belongs to a family of pipecholyl xylidines. Initially the safety profile was very encouraging when separation between motor anaesthesia and sensory anaesthesia was produced. However, severe cardiovascular toxicity of bupivacaine was reported after ten years of use in clinical practice.



Structure of Bupivacaine

In 1979 George Albright published a shocking editorial in which he highlighted reports regarding cardiac arrest caused by bupivacaine and etidocaine used during regional anaesthesia. Six cases of simultaneous convulsion along with sudden cardiac arrest were reported by Albright which required a prolonged and mostly unsuccessful recovery after a presumed intravascular injection of bupivacaine or etidocaine illustrating the narrow margin that exists between efficacy and cardiac and cerebral toxicity [19].

Later on in October 1983 Albright presented his findings to the FDA in which he reported a detailed series of 49 cases of ventricular tachycardia needing cardioversion that had happened over the past ten years resulting from the use of bupivacaine. These cases mostly were during obstetric epidural anaesthesia that used 0.75% bupivacaine. This information led the FDA to issue an urgent sanction on the use of 0.75% bupivacaine in obstetrics [21].

At the same time in the United Kingdom, anaesthetists started a campaign to stop the use of bupivacaine during IV regional anaesthesia (IVRA) because five deaths were reported related to bupivacaine from 1979 to 1982. According to an editorial that was published in 1982 in the British Medical journal [23] all five patients were healthy and

being treated with bupivacaine with IVRA administration in an emergency department for minor conditions. The deaths still occurred although the drug was given according to the recommended dosage and procedures. This led to the abandonment of the use of bupivacaine during IVRA but still it remained the cause of deaths because of unintended IV administration. Three deaths were reported in UK up to 2004 because of accidental administration of bupivacaine intravenously. During the development of new amides and long acting local anaesthetics in 1980s, scientists began to consider that there was a chiral centre in most of this class of molecules. These chiral centres in the new amide long acting local anaesthetics had significant safety implications because the levorotatory(S) isomer was found to have less risk of systemic toxicity as compared to the dextrorotatory isomer. So on the basis of these observations new single stereoisomer versions of these anaesthetics, ropivacaine and levobupivacaine, have been developed and were first approved in 1996 for clinical use in North America [21, 24, 25].

2.1.2 Ropivacaine

Ropivacaine is the newest type of a long acting amide type local anaesthetic and along with levobupivacaine is used for regional anaesthesia. Ropivacaine is closely related to bupivacaine both in chemical structure and also in anaesthetic action. In contrast to other long acting amide type anaesthetics, ropivacaine was produced and marketed from the outset as the pure -S- enantiomer. As it was found that the cardiotoxicity of the S configuration of the the drug was lower than that of the R configuration, also the

degree of motor blockade by ropivacaine is less than that of bupivacaine although both the agents causes the sensory blockade up to same degree. These findings made ropivacaine a superior local anaesthetic agent to bupivacaine [24].

2.1.3 Structure activity relationship

All local anaesthetics in clinical use generally consist of three parts, a lipophilic or aromatic part, a hydrophilic or amine part and a bond or link between these two parts. The linking bond may be either an ester or an amide [26]. Ropivacaine is prepared as the S enantiomer and is very similar in chemical structure to bupivacaine and mepivacine. Generally amide local anaesthetics are termed as chiral compounds because of the asymmetric carbon atom in their structures. Thus these compounds exist in two forms like mirror images of each other or enantiomers (figure 2.1 and 2.2). The left and right segments of these enantiomers are distinguished from each other by S (sinister) and R (rectus) terms. These enantiomers change the path of plain-polarized light either to left or right direction and referred as l or d (- or +). Local anaesthetics were synthesized as racemates in the past containing equal portions of both the configurations but nowadays it is possible to chemically synthesise single pure enantiomers for research purposes. Ropivacaine and bupivacaine both are related to mepivacaine by substituting the methyl group ($-CH_3$) attached to the nitrogen with a propyl group ($-C_3H_7$) for ropivacaine and a butyl group ($-C_4H_9$) for bupivacaine. Thus the difference between ropivacaine and bupivacaine and mepivacaine is only the carbon side chain length attached to the tertiary nitrogen atom. By varying the carbon chain

length, the lipid solubility also changes and ultimately affects the potency of the various local anaesthetics. The 3D orientation of L- and S- enantiomers is different across the asymmetric carbon which causes changes in the biological activities of different compounds. For example cardiac sodium channels might be stereo-selectively helpful in the uptake of R-configuration for local anaesthetics and this could give an explanation for the enhanced cardiotoxicity related with R-configuration. The advancement in both extraction techniques and stereo-selective synthesis helped scientists in the production of local anaesthetics as a single enantiomer commercially, as a result ropivacaine is produced which at the time of its marketing has more than 99% of the S-form [24].

Figure 2.1 Enantiomers are like right and left hand gloves.

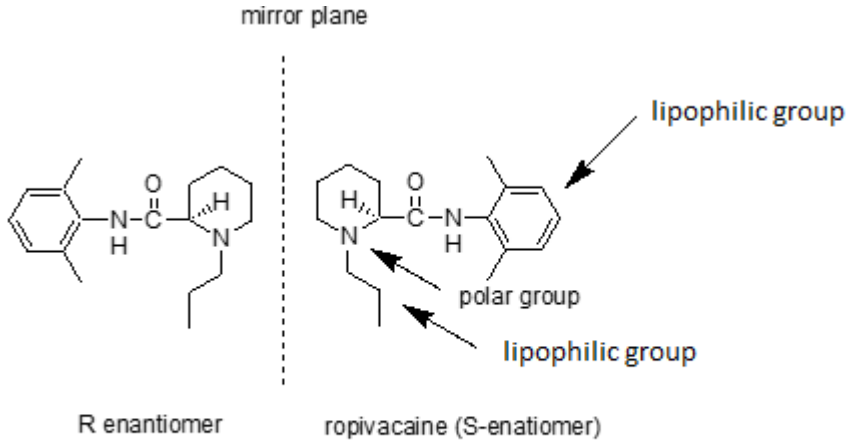


Figure 2.2 Structure of ropivacaine showing chiral centre and polar and lipophilic regions of the molecule.

2.1.4 Pharmacodynamic properties

Ropivacaine has similar pharmacodynamic properties to other members of the amide local anaesthetic class. This section gives an overview about ropivacaine's mechanism of action and its pharmacodynamic effects [18].

2.1.4.1 Mechanism of action

Ropivacaine acts by reversibly blocking the conduction of the nerve impulse by reducing the permeability of the nerve cell membrane to sodium ion influx. Nerve fibres may be myelinated (surrounded by a lipophilic layer) A and B or nonmyelinated C. Aδ and C fibres are responsible for the transmission of pain while Aα and Aβ fibres control motor functions. Amide local anaesthetics generally have moderate pKa values (*ca* p*K*a 8) and moderate lipid solubility in their ionised form therefore causing blockade of C fibres preferentially over A fibres. According to an isolated animal nerve study, ropivacaine was shown to be more selective for Aδ and C (pain) fibres than Aβ motor fibres [17, 18]. Ropivacaine has pKa value of ≈8.2, similar to bupivacaine but is less lipid soluble than bupivacaine because of having a shorter alkyl chain attached to the tertiary nitrogen group and is therefore less likely to enter myelinated motor nerve fibres [17].

Rosenberg and Heinonen in 1983, showed that ropivacaine is more potent in low concentrations by producing a rapid blockade of Aδ and C fibres as compared to bupivacaine in a similar concentration. However the blocking activity is similar for both the drugs in higher concentrations. In another study on the isolated vagus nerve of rabbit, by comparing high doses it was shown that ropivacaine blockade of motor fibres was 16% less than that of bupivacaine in similar concentration but had the same effect on sensory fibres in these concentrations. Wildsmith *et al*. used a desheathed vagus nerve of a rabbit to show that ropivacaine blocked C fibres more quickly than A fibres

and was a very potent frequency dependent blocking agent i.e. the blockade was produced after stimulation of the fibre. Frequency dependent blockade is thought to be associated with lipid solubility and the molecular weight of the drug. Thus ropivacaine with a high degree of differential blockade in low concentrations and the ability to produce frequency dependent blockade and a minimal motor block presents significant clinical advantages in providing analgesia [25].

2.1.4.2 Cardiovascular and central nervous system (CNS) effects

Cardiac adverse effects associated with long acting anaesthetics are significantly higher risk for patient's lives. A number of studies suggest that death associated with local anaesthetic toxicity is primarily because of cardiac arrhythmia and cardiovascular depression [27]. Ropivacaine like other local anaesthetics produces toxic cardiovascular and CNS effects after higher doses or unintentional intravascular administration of the drug. Cardiovascular toxicity occurs at higher plasma concentrations than those provoking CNS toxicity. Ropivacaine has higher threshold for cardiovascular and CNS toxicity as compared to bupivacaine because of its lower liphophilicity and its stereoselective properties. Cardiotoxicity related to local anaesthetics is believed to occur because of two reasons either slow dissociation of drug across cardiac sodium channels resulting in channel blockade and/or impairment in the metabolism of cardiac mitochondria, according to two different studies. Ropvacaine causes less disturbance in cardiac mitochondrial energy metabolism than bupivacaine. Potassium channel blockade is also responsible for cardiotoxocity by increasing the duration of cardiac

action potential; a limited amount of evidence also suggests that the affinity of ropivacaine for the human potassium channel is lower than that of bupivacaine. In a study on anaesthetised pigs it was demonstrated that cardiotoxic potential related to ropivacaine was half that of bupivacaine and levobupivacaine and ropivacaine also caused less prolongation in QRS duration than bupivacaine. Ropivacaine also had approximately a 50% higher lethal dose than bupivacaine [17]. In animal studies cardiodepressant activity associated with ropivacaine was less than that of both the isomers of bupivacaine because of its lower lipophilicity. Also bupivacaine (both isomers) was responsible for longer atrioventricular conduction time than ropivacaine. The inotropic and depressant effect of ropivacaine was also significantly lower than that of bupivacaine. The lesser lipophilic property of ropivacaine is also very important because it causes less depression of synthesis of mitochondrial adenosine triphosphate (ATP) in fast metabolising cells and according to in vitro and in vivo data reported in the literature, the intracellular level of ATP is responsible for contractility as well as resuscitation of cardiomyocytes [18]. This study, found that three out of four dogs recovered after induced ventricular dysrhythmias after receiving ropivacaine but none recovered after receiving bupivacaine [18].

2.1.4.3 CNS effects

The central nervous system is one of the foremost target systems of local anaesthetics for toxicity [27]. The toxicity of local anaesthetics in the CNS appears to be a two-step process, an excitatory stage at low concentration, because local anaesthetics have

affinity for inhibitory neurons leaving the excitatory neurons to act in an unrestricted way producing an excitatory stage. This is then followed by depression of the CNS in high concentrations affecting all neurons and leading to serious effects, which include convulsions, generalized CNS depression and ultimately leading to coma because of zero EEG and cardiovascular collapse. Signs of systemic poisoning can also be seen with continuous or intermittent infusion of the drug but the toxic limits vary depending on the patient's age, disease type and the speed of the infusion [27]. However according to a double blind study, the threshold for CNS toxicity was significantly higher for intravenous ropivacaine as compared to bupivacaine. Similarly in another study it was shown that bupivacaine possessed greater toxicity and the severity of CNS symptoms was greater than that of ropivacaine in healthy volunteers [17].

2.1.5 Therapeutic applications of ropivacaine

2.1.5.1 Infiltration anaesthesia

Intraoperative wound infiltration nowadays is commonly used in patients of all ages for the relief of the pain immediately after surgery. The rate of regional blood flow determines the action of a specific agent both at the injection site and the systemic concentration. Sometimes a local vasoconstrictor agent is also given to enhance the effect of an anaesthetic agent and to prevent toxic plasma levels. According to an experimental study on pigs [28], ropivacaine had a vasoconstrictive effect itself in low concentrations after subcutaneous injection. Similarly it had a vasoconstrictive effect on the canine femoral vessels *in vitro*, however, bupivacaine on the other hand had a

vasodilation effect in all concentrations. The effect of ropivacaine was also longer after intradermal injection than bupivacaine in equal doses possibly because of its vasoconstrictor effects. These findings that bupivacaine caused vasodilation whereas ropivacaine was responsible for vasoconstriction at the injection site were also confirmed from studies in human volunteers comparing the effects of both the drugs on cutaneous blood flow [29]. Ropivacaine is also thought to exhibit a biphasic effect on human vasculature having no vasoconstriction properties when used as a highly concentrated solution. However, vasoconstriction produced at lower concentration was believed to have a longer duration of action for ropivacaine. The duration of action of both the drugs was increased by adrenaline but the ropivacaine was thought to decrease the vasoconstricting action of adrenaline. However, there were some doubts about these clinical findings as some researchers thought that cutaneous vasoconstriction was related to the very small dose in the experimental set up and there would be no significant relation to vasoconstriction with doses used for clinical purposes [30]. There were also concerns raised about infiltrating ropivacaine into end-arterial blood supply areas. There was one report of temporary ischaemia of the glanspenis in which the penile block was observed after 40 minutes of administration of 0.75% w/v ropivacaine, so the use should be avoided if there is a risk of ischaemia to end organs. Several studies were carried out in patients to show the effects of ropivacaine after wound infiltration for the relief of post-operative pain. All these

studies concluded that pre-operative infiltration of ropivacaine markedly reduced the pain after the surgery [27].

2.1.5.2 Peripheral nerve blocks

Several studies on isolated nerves of animals compared conduction block caused by ropivacaine, bupivacaine and levobupivacaine in similar concentrations, and these studies confirmed that the onset and duration of the blockade produced by all these drugs were similar [31]. A number of different studies [32] also confirmed these findings by comparing ropivacaine with other local anaesthetics for peripheral nerve block. These studies suggested ropivacaine produced nerve blockade that was similar to bupivacaine and levobupivacaine in equimolar doses. However, a short onset time and prolonged duration of nerve block was observed by increasing the dose and concentration of ropivacaine as compared to bupivacaine and levobupivacaine. Cline *et al* [33] found that sensory analgesia produced by levobupivacaine in similar concentrations was longer than that of ropivacaine in combination with epinephrine (1:200,000) while studying the effect of both the drugs for axillary brachial plexus block. However, the recovery of motor functions was faster in patients receiving ropivacaine. In different studies comparing the use of ropivacaine levobupivacaine and bupivacaine for blocking the sciatic nerve for foot surgery, it was found that the effects of these drugs were similar at 0.5% w/v concentration, however, at 0.75% w/v concentration levobupivacaine produced a shorter onset time and longer analgesic effect after the surgery as compared to 0.75% w/v ropivacaine, these findings were

supported by Piangatelli *et al* [34] in patients having surgery of lower extremities when they compared 0.5% w/v levobupivacaine and 0.75% w/v ropivacaine for blocking sciatic nerves and compared the clinical effect on the psoas muscle.

In another study, postoperative analgesia produced by ropivacaine was compared with that of bupivacaine for peri-neural infusion in low concentrations. It was shown that ropivacaine at 0.2% w/v produced the same relief in pain compared to 0.15% w/v of racemic bupivacaine. Similarly in another study Casati *et al* [35] compared inter-scalene analgesia produced by 0.2% w/v ropivacaine and 0.125% w/v levobupivacaine in patients undergoing major shoulder injury. They found that both the drugs produced the same analgesic effect after the surgery and motor recovery, although the consumption of both the drugs was different during the first 24 hours. Borghi *et al* [36] also compared levobupivacaine 0.25% w/v with ropivacaine 0.25% w/v and 0.4% w/v and found no difference in pain relief quality, motor function recovery or the number of boluses required in patients receiving levobupivacaine and 0.4% w/v ropivacaine. However, the patients receiving 0.25% w/v ropivacaine needed a greater number of boluses to produce the same degree of postoperative analgesia. Casati *et al* [37] also carried out a study in which they compared ropivacaine and levobupivacaine and showed the continuous blockade of popliteal sciatic nerve with these two drugs. They compared a 0.2% w/v concentration of ropivacaine with either the same 0.2% w/v or equipotent 0.125 % w/v levobupivacaine. The results suggested that there was no difference in the degree of pain relief either in rest or in motion, but the total recovery

of motor functions of the foot was less frequent as compared to patients receiving ropivacaine 0.2% w/v and levobupivacaine 0.125% w/v.

2.1.5.3 Epidural anaesthesia and analgesia

The historical background of ropivacaine, less cardiotoxic properties and less intense motor blockade and shorter duration of action than bupivacaine, has made ropivacaine an ideal agent for epidural use. Its use is also desirable when motor functions of lower limbs have to be preserved. Ropivacaine has been widely studied as analgesic for obstetric use where it has shown significant advantages in comparison to bupivacaine [38].

Different studies suggested that both of these drugs were equally effective in pain relief when use in same concentration but closer analysis of these studies showed that ropivacaine was more effective than bupivacaine in epidural block since it allowed for more spontaneous vaginal deliveries, less instrumental deliveries as well as an enhanced neonatal outcome score [39]. However, the relative potencies of ropivacaine and bupivacaine have been questioned in recent studies. Columb and Lyons in 1995, firstly used an up and down method for comparing the minimum local analgesic concentration (MLAC: the median effective concentration in 50% patients (EC50)) of ropivacaine and bupivacaine and it has been suggested that the potency of ropivacaine may be 40% less than that of bupivacaine. The study aimed to determine the effective concentration of both agents for 50% of patients or simply the “ED50” using a predetermined volume of each anaesthetic. The results obtained in this study have

been employed to support an argument that the advantage of reduced motor block as well as reduced cardiotoxicity for ropivacaine over bupivacaine must be balanced against the obvious decrease in the potency. However, a number of researchers have raised their concerns about this method for determining the potency of local anaesthetics. The first question was about the relevance of the figure obtained as only half of the patients benefited. The second question raised was about the concentration of the drug, in which the results were expressed which changes with time. The third point was about the shape of the dose response curve which was obtained only for one data point, thus it was suggested that without prior studies for describing the dose response curve for both the agents it is impossible to obtain the shape of the curve on the basis of single data point. Thus the results of MLAC studies contradicted the results of those clinical studies where all the patients got pain relief. So it was concluded that both the agents are responsible for same degree of pain relief in the same concentration and there are other important advantages with ropivacaine [38].

2.1.5.4 Spinal anaesthesia

Ropivacaine has not been commonly used for spinal anaesthesia. Two different studies involving intrathecal injection of ropivacaine were carried out in order to ensure that there would be no adverse effects in cases of unintentional intrathecal injection. The injections produced sensory block of intermediate duration and unpredictable extent [38]. Ropivacaine is not currently approved for its use in spinal anaesthesia because of the lack of reliable clinical data; the focus of the research to date is mainly on the

safety profile and dose related matters of the drug [27]. In a recent study comparing ropivacaine with bupivacaine, Gautier *et al* [40] used large volumes of less concentrated solutions than normal solutions of glucose free preparations, and they observed no difference in the onset as well as the extent of sensory block. However, they found that ropivacaine produced a shorter duration of sensory blockade and less degree of motor blockade; these findings suggest that the potency of ropivacaine is less. However the interesting thing is that the patients taking ropivacaine was found to become mobilized and passed urine more quickly than patients receiving bupivacaine [27, 38].

McDonald *et al* [41] conducted a study on volunteers who were not undergoing surgery in which they compared the effect of intrathecally injected hyperbaric ropivacaine and bupivacaine. Less concentrated solutions than normal was used in the study. The results showed that sensory block produced by both the drugs were similar in terms of onset and extent but the motor block was shorter with ropivacaine. They also observed high rate of backache and side effects with ropivacaine, so they concluded on the basis of the shorter duration of action that ropivacaine is less potent as compared to bupivacaine. However, the subclinical dose used in these studies needs to be addressed and needs more interpretation.

In contrast to the above studies some recent work was carried out in both the obstetric and non-obstetric population. The results showed excellent spinal blockade after injecting hyperbaric ropivacaine. So it was concluded that with proper concentration

and doses a more appropriate block profile could be obtained in the relevant surgical procedures [38].

2.1.5.5 Use of Ropivacaine in the Management of labour pain

The use of ropivacaine for relief of labour pain through the lumbar epidural route is well established. Regarding analgesia and motor block there was not much difference found between ropivacaine and bupivacaine in epidural ropivacaine trials although it was shown that in achieving analgesia, in about 50% women ropivacaine might be about 25% less potent than bupivacaine. The volume as well as concentration of ropivcaine has no significant effect on the efficacy of ropivacaine if used epidurally for labour analgesia. In one double blind study it was shown that epidural infusion of ropivacaine at 0.25% w/v concentration was responsible for a shorter duration in the first stage of labour than bupivacaine (0.25% w/v) although there were no other differences observed. Another study showed that epidural administration of a 15 ml bolus of 0.0625% w/v ropivacaine, bupivacaine or levobupivacaine along with fentanyl in nulliparous women provided sufficient analgesia with not other significant differences, fentanyl can also increase the duration of analgesia and can be correlated with an increased satisfaction of patients [42].

2.1.6 Pharmacokinetics

2.1.6.1 Absorption and distribution

Generally if the local anaesthetic is more quickly absorbed from the injection site, then the block duration is also shorter and there are more chances of systemic toxicity [24].

The concentration of ropivacaine in the plasma is determined by the total dose administered, the rate of the administration, the injection site and also on the vascularity of the site of administration of the drug. The rate of dissociation of local anaesthetics from proteins and adipose tissue also has some influence on systemic absorption.

Amide local anaesthetics are classified as weak bases and are considered to be comparatively lipophilic drugs thus exhibiting a biphasic pattern for distribution. According to literature [24] biphasic distribution means that initially ropivacaine start distributed rapidly to areas with a high blood supply, also known as the α phase which is then followed by a slower or β phase where ropivacaine distributes slowly into less vascularised areas like muscles and fats. The rate of absorption influences Cmax (Cmax is the maximum concentration of drug measured in the body) and Tmax (Tmax is the time take to reach Cmax in the body) and can be affected by the presence of vasoconstrictors, however, these two parameters (Cmax and Tmax) are considered as very important parameters as they depend primarily on when samples of the blood are taken. Ropivacaine after reaching the systemic circulation is bound primarily to α 1-acid glycoprotein (AGP), which is an acute phase protein [24]. There is an increase in the

plasma concentration of ropivacaine after continuous epidural infusion because increased protein binding of the drug is followed by a decrease in the clearance of ropivacaine. The variation in protein binding of ropivacaine is associated with an increase in concentration of α 1-acid glycoprotein [18], the levels of which are increased in stress and other surgical conditions resulting in a varied total plasma concentration of the protein [18, 24], plasma protein binding is discussed in detail in chapter 3. After intravascular administration of ropivacaine the volume of distribution was 41L at steady state [18]. According to a review by Hansen [24], ropivacaine is likely to accumulate in the body if the rate of infusion exceeds the biotransformation and elimination rate of the ropivacaine. Although the total ropivacaine plasma concentration increases with time when using it as an infusion for analgesia after surgery, the free portion of the drug still remains within limits. This is because of the related increase in the concentration of AGP caused by surgical trauma [24]. Ropivacaine also readily crosses the placenta if it is administered as an epidural injection for caesarean section and it reaches the fetal circulation where equilibrium is established quickly for the free drug. However, the total concentration of ropivacaine in the plasma is less in fetal blood than in the maternal circulation because the AGP is more concentrated in maternal blood [18].

2.1.6.2 Metabolism and elimination

Like bupivacaine the amide bond in ropivacaine is shielded by two methyl groups which are ortho to it in the aromatic ring and it is therefore protected from hydrolysis.

Metabolism eventually occurs in the liver by hepatic microsomal cytochrome P450 (CYP). Hepatic metabolism of ropivacaine mainly depends on the blood flow to liver as well as the extent of protein binding [24]. The predominant metabolism of ropivacaine is by aromatic hydroxylation converting ropivacaine to 3'-hydroxy-ropivacaine by CYP1A2 and by N-dealkylation to the metabolite 2', 6'-pipecoloxylidide by enzyme CYP3A4. Minor metabolites produced are 4-OH-ropivacaine and 2-OH- methyl ropivacaine [18]. Drugs that inhibit the activity of these enzymes, particularly CYP1A2, influence the pharmacokinetic parameters of ropivacaine, for example fluvoxamine if given with ropivacaine will increase the total concentration of ropivacaine in the plasma after intravenous injection by reducing its clearance [24]. After intravenous injection of single dose of radiolabelled ropivacaine about 86% of the ropivacaine was excreted as 3'-hydroxy-ropivacaine in the urine whereas about 1% of the parent drug was excreted as unchanged [18]. According to [25] the elimination half life of ropivacaine was 111 ± 62 minutes.

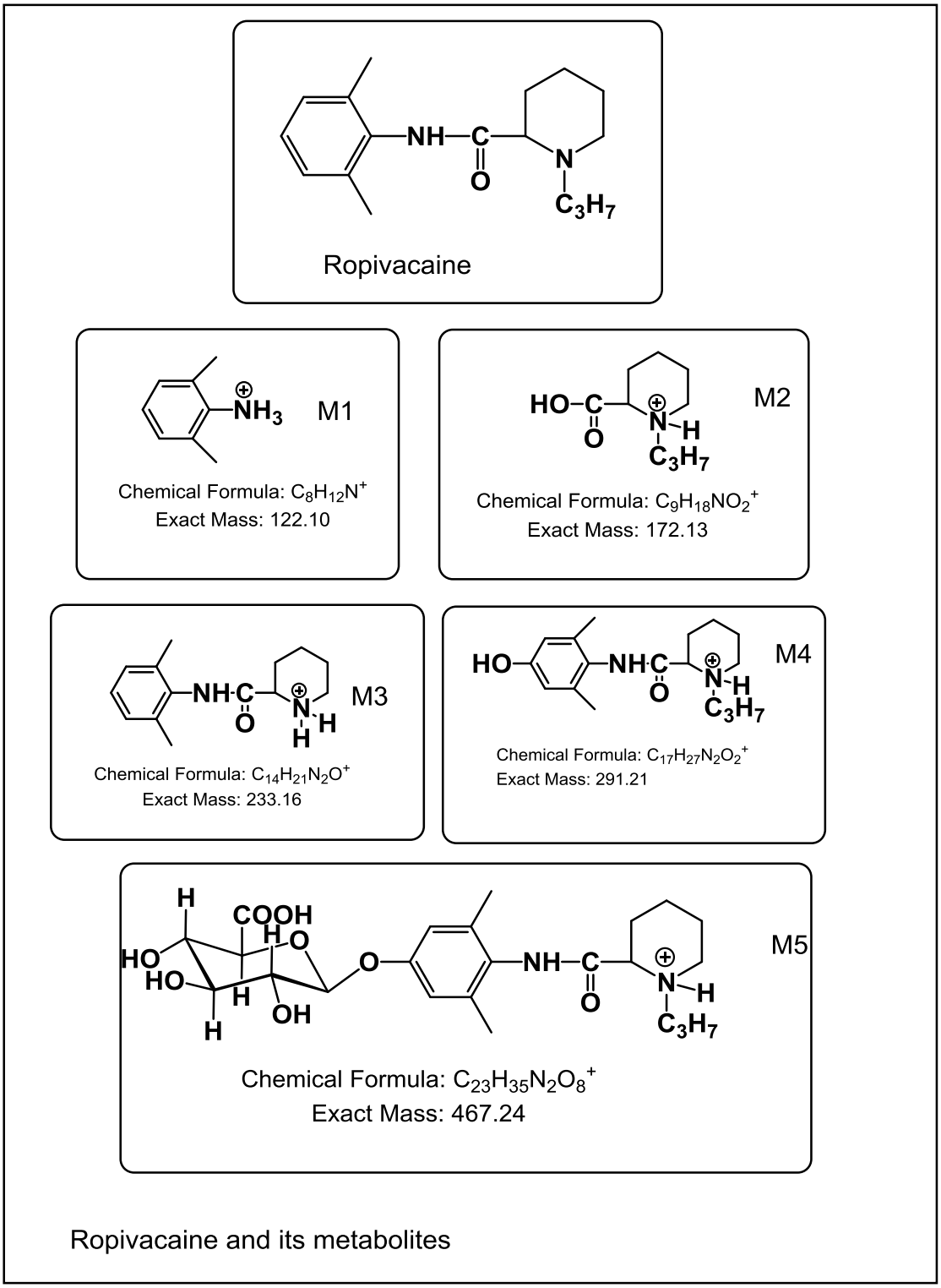


Figure 2.3 Ropivacaine and its possible metabolites

2.1.6.3 The effect of pH on anaesthetic activity

Local anaesthetic agents are weak bases having pka values of 7.6-8.9 and can be prepared as acidic hydrochloride solutions (pH 3-6) because they are not readily soluble in water. Local anaesthetics exist in solution either as unionised base or neutral base and a cationic moiety [43, 44]. The unionised base rapidly diffuses across the cell membrane; the quantity of the unionised form of local anaesthetics in solution depends on the pKa and pH of the solution [45]. The Henderson–Hasselbalch equation can be used to determine the proportion of each form.

pH = pKa + log[base]/[acid]

In the case of a base

pH = pKa + log[unionized]/[ionized]

The proportion of each form depends on the pKa of the anaesthetic. As the pKa is almost constant for every local anaesthetic it is the pH of the solution that determines the quantity of each form in the solution. All local anaesthetics have pKa values from 7.6-9.1 except for benzocaine, which are greater than the pH of physiological fluids at pH 7.4 and therefore at the injection site most of the drug is present in charged form. The speed of onset is increased for drugs with low pKa values because most of the drug is present in unionized form that enters the cell. So the pKa of the anaesthetic is an important factor governing the onset time [43, 44, 45].

The pH of the physicological fluid that the anaesthetic is dissolved in is an important determinant of the quantity of unionized local anaesthetic that can cross the cell

membrane [45]. Since local anaesthetics are weak bases they are stored and supplied in acidic solution with a pH normally from 5 to 6 to facilitate solubility and also to prevent precipitation at higher pH. The low pH is also responsible for greater physical stability [45, 46]. A more rapid onset of conduction blockade was observed by increasing the pH of the injection solution, because of the availability of a greater quantity of the unionised and lipid soluble form of the drug making it more available to cross cell membranes and ultimately reach the site of action. However too much alkalinity can lead to precipitation of the drug and that can reduce the bioavailability as a result [46]. Many anaesthesiologists have used sodium bicarbonate for alkalinization of anaesthetic solution to promote the rapid onset of blockade however quantity of sodium bicarbonate required for local anaesthetic solutions varies with specific type of local anaesthetic [45]. The pKa (dissociation constant) of ropivacaine is 8.1 and similar to that of bupivacaine. A study [47] was conducted to show the effects of pH on the precipitation of ropivacaine solution involving 0.2% w/v ropivacaine solution and increasing strengths of bicarbonate. The results concluded that the effect of ropivacaine alkalisation with bicarbonate was similar to the effect on bupivacaine; the results also showed that 0.1 ml of 8.4% of sodium bicarbonate was the optimal amount for the alkalisation of 20 ml 0.2% ropivacaine solution. Thus, clinically, with this kind of solution prepared just before the epidural injection, rapid onset of anaesthesia was induced with less chance of a decrease in bioavailability. Although it was shown that the higher pH was responsible for rapid onset of blockade by enhancing the

concentration of unionised local anaesthetic, the exact mechanism for this action is still unknown. Ackerman *et al*. [48] conducted a study in which they used a buffer called tromethamine to increase the pH of an anaesthetic solution for epidural block but they observed that there was no rapid onset of blockade thus they suggested that bicarbonate may possibly act by carbonation i.e. by CO_2 changes. In carbonation, CO_2 is added to the local anaesthetic for the rapid onset of nerve blockade. CO_2 also may act independently either by depressing nerve membranes directly or rapidly entering into the nerve cell where it decreases the intracellular pH thus enhancing the onset of nerve blockade. As a result this would enhance the cationic form of the anaesthetics intracellularly thus leading to the increased binding of the drug to sodium channels.

2.1.6.4 The effect of local anaesthetics on blood

Amide type local anaesthetics have been shown to decrease the clotting of blood. This reduction in blood clotting is concentration dependent and would be either by directly hindering platelet function or by increasing the process of fibrinolysis. Ropivacaine also has been found to be responsible for altered functions of platelets like other amide anaesthetics [46].

2.1.6.5 Lipid solubility

The potency of a local anaesthetic can best be determined by its lipid solubility, the more lipids soluble the local anaesthetic is, the higher will be its potency for reaching the target site [45]. The nerve membrane consists of different layers of lipids so local anaesthetics have to cross these lipid layers in order to reach the inner portion of the

sodium channels in the cell. So lipid solubility is an important determinant of the ability of the local anaesthetic to pass through the membrane. The lipid solubility of a local anaesthetic can be determined by its relative distribution in the aqueous phase and organic phase, this distribution in the aqueous and organic phase enables determination of partition coefficient. The greater the partition coefficient of a substance, the higher will be its lipid solubility and it will cross the cell membrane more rapidly and easily to reach the target receptor. The aromatic group along with side chain hydrocarbon affects the lipid solubility of the local anaesthetic and has been related with the potency of the local anaesthetic [43, 44].

2.1.7 Ropivacaine in children

Ropivacaine binds to α1- acid glycoprotein (AGP) in the plasma and the concentration of AGP in neonates and infants is lower in infants than in adults, and also there are differences in the functioning of major organs responsible for metabolism of ropivacaine. For these reasons infants and neonates are more prone to toxicity from ropivacaine administration. Some preliminary studies in children have determined the safety as well as the pharmacokinetic and pharmacodynamic parameters of ropivacaine administered via epidural or caudal injection. In a study determining the safety of ropivacaine in children 1-8 years old, a total of 1 ml/kg of 0.2% w/v ropivacaine was administered as a single shot caudal injection. The results indicated that the total bound and free portions of ropivacaine were below the toxic level. The weight corrected values were determined for clearance of the drug, volume of distribution and

elimination half-life and no difference was found in these values in children aged 1- 12 years. However, in infants aged less than 3 months a relatively highly proportion of free of ropivacaine was observed after caudal injection [46]. In a study [49] comparing the pain relief produced by 0.25% w/v ropivacaine and 0.25% w/v bupivacaine in patients undergoing urological and lower abdominal surgeries, no difference was found in the degree of pain relief. Similar results regarding pain relief were obtained in another group of children (3-6 years) where ropivacaine and bupivacaine were both used in a concentration of 0.375% w/v [50]. Similarly the extent of analgesia after caudal injection produced by both ropivacaine and bupivacaine at a concentration of 0.25% w/v were similar in hernia repair in children aged 2-5 years [51]. The most important point in all studies was that less motor blockade was associated with ropivacaine as compared to bupivacaine. One of the studies involved infants aged less than 1 year and children aged 1-5 years who were given ropivacaine through caudal injection for inguinal hernia repair, and although high levels of ropivacaine appeared in plasma no signs of toxicity were observed and the block produced was highly satisfactory. Luz *et al* [52] also suggested that caudal analgesia produced with 0.2% w/v ropivacaine was related to significantly less motor blockade after the surgery as compared with bupivacaine in the same concentration. Hansen *et al* [53] showed in his review that no adverse reaction was found when ropivacaine was infused at a rate of 0.4 mg/kg per hour for long term (36- 48 hours). He found that the total and free concentrations of ropivacaine in this study were below the adult limit that was responsible for side

effects. A previous study showed that the maximum free concentration of ropivacaine in infants aged less than 2 months was 0.215 µg/ml so on the basis of these results Hansen suggested that the epidural infusion should not be used for more than 36 to 48 hours in young children because of the limited capacity of AGP binding of the drug in young patients. Also the use of 0.5% w/v ropivacaine at a rate of 0.6 ml/kg in paediatric patients of age 1-2 years for peripheral nerve blocks was determined to be safe. Similarly 0.2% w/v ropivacaine solution was used for popliteal fossa block and was found very effective in pain relief. The drug was given at a rate of 1.5 mg/kg in patients aged 0.5-12 years undergoing foot surgery. In short, ropivacaine was found to have a similar profile regarding efficacy, onset time and extent of analgesia in children as compared to bupivacaine but less motor blockade was associated with ropivacaine [46].

2.1.8 The local infiltration analgesia technique

Local infiltration analgesia or simply LIA is an intraoperative administration technique for local anaesthetics in combination with other drugs like NSAIDs (Nonsteroidal anti-inflammatory drugs), opioids and steroids for the relief of postoperative pain after total knee (TKA) and total hip arthroplasty (THA). In this technique there is placement of a catheter in a wound for continuous infusion of local anaesthetics or analgesics postoperatively in order to provide analgesia for a longer period of time [54]. The technique of LIA was first introduced by Bianconi and his colleagues, Kerr and Kohan [55]. They got some good early results from their observational non- randomized study

which included 325 patients and they achieved an excellent control of the pain as well as early discharge of patients from hospital. The interest of anaesthesiologists in using the technique of LIA to control post-operative pain has increased in recent years. Andersen and Kehlet [54] conducted a review of randomised clinical trials in which they investigated the efficacy of local infiltration analgesia (LIA) in total hip and knee arthroplasty for the relief of pain after surgery. Similarly efficacy of wound catheters and the length of hospital stay after TKA and THA were also evaluated. The review included 27 randomized trials including 756 patients operated on for THA and 888 patients operated on for TKA. In THA patients there was no additional analgesic effect observed when LIA was compared against placebo in those trials with a lower chance of bias through the administration of multimodal analgesic regimen perioperatively. LIA was shown to have either the same or enhanced analgesic effect when compared against intrathecally administered morphine and epidural analgesia. Similarly in TKA the LIA technique produced more relief of pain and also reduced the opioid requirements in most of the trials as compared to placebo or no injection. Local infiltration techniques also provided the same or a high degree of analgesia as compared to blockade of the femoral nerve or epidural/intrathecal morphine. However, there was an increased chance of bias because of the difference in systemic analgesia used between different groups. The use of wound catheters and length of hospital stay was shown to be not much related to local infiltration analgesia. So

according to these trials LIA was found to produce effective analgesia in the early period after surgery [54].

2.1.9 Dosage and administration

Ropivacaine is used both in adults and children for various clinical procedures. In adults it is indicated for surgical anaesthesia through the epidural or intrathecal routes, blocking peripheral nerves or by cutaneous infiltration. For the relief of postoperative pain it is administered through the epidural route, through wound instillation or by peripheral nerve blockade. In labour or to relieve other types of acute pain it is administered through the epidural route. Ropivacaine is also used in children aged 1-12 years and is administered epidurally as a continuous infusion and for blocking peripheral nerves for the management of acute pain or postoperative pain. Ropivacaine is generally prepared as 0.2%, 0.5%, 0.75% and 1% w/v solutions and is available in ampoules of 10 ml and 20 ml for injection and a 0.2% w/v solution is available in infusion bags of 100 ml and 200 ml. The dose of ropivacaine depends on its intended use, for example for surgical anaesthesia higher doses are needed as compared to labour analgesia or for the relief of postoperative pain. When using ropivacaine as a continuous infusion or as a bolus doses for prolonged blocks and for blocking peripheral nerves (where the drug is normally injected near to major blood vessels) the plasma concentration should be taken into consideration because of the risk of it reaching to toxic levels. Ropivacaine is recommended to be given in incremental doses to lower the risk of overdose. The approved dosage regimen of

ropivacaine is different in different countries, therefore the local prescribing information for the use ropivacaine should be referred to [18]. Ropivacaine dosages according to route of administration and procedures are listed in table 2.1.

Table 2.1 Ropivacaine dosage regimens in adults and children

Indications and procedure	Conc (% w/v)	Vol (mL)	Dose (mg)
Adults			
Surgical anaesthesia			
Lumbar epidural (Caesarean section)	0.75	15-20	113-150
Lumbar epidural (other surgery)	0.75	15-20	113-188
	1	15-20	150-200
Thoracic(single block for postoperative pain relief)	0.75	5-15	38-113
Intrathecal administration	0.5	3-4	15-20
Peripheral nerve (Major nerve block,brachial plexus)	0.75	10-40	75-300
Field block (minor nerve block or infiltration)	0.75	1-30	7.5-225
Postoperative pain			
Lumbar epidural (continuous infusion)	0.2	6-10¤	12-20*
Thoracic epidural (continuous infusion)	0.2	6-14¤	12-28*
Peripheral nerve block (continuous infusion)	0.2	5-10¤	10-20*
Field block (minor nerve block or infiltration)	0.2	1-100	2-200
Intra-articular injection	0.75	20	150
Labour pain (lumbar epidural)			
Bolus	0.2	10-20	20-40
Intermittent top-ups	0.2	10-15	20-30
Continuous infusion	0.2	6-14¤	12-28*
Children			
Caudal epidural block (body weight up to 25kg)	0.2	1¤¤	2**
Peripheral nerve block (e.g. illioinguinal nerve block)	0.5	0.6¤¤	3**

Note: ¤ mL/h; ¤¤ mL/kg; * mg/hr, ** mg/kg

2.1.10 Literature review about determination of ropivacaine in human plasma

A number of research papers are available regarding the determination and analysis of ropivacaine in blood, plasma, urine etc. Mathieu *et al* determined the concentration of free and bound portions of ropivacaine in plasma obtained from infants who were undergoing either epidural anaesthesia or continuous psoas blockade by using equilibrium dialysis and liquid chromatography coupled with an electrospray ionization mass spectrometry system [56]. Sawaki *et al* determined the concentration and other pharmacokinetic parameters of ropivacaine in plasma obtained from rabbits by liquid chromatography mass spectrometry (LC/MS) using a reverse phase column in their study [57]. A paper was published by Salmeron-Garcia *et al* regarding the determination of ropivacaine along with three other drugs bupivacaine, tramadol and metamizole in samples of analgesic mixtures which were used in patient controlled analgesia by using HPLC with Diode array detection (DAD) [58]. Similarly Koehler *et al* developed a method for simultaneous determination of ropivacaine along with three other local anaesthetics bupivacaine, mepivacaine and prilocaine in serum. The method was developed on a liquid chromatography-tandem mass spectrometry system [59]. Abdel-Rehim *et al* determined ropivacaine and also the metabolites of ropivacaine in patient urine by using liquid chromatography coupled with tandem mass spectrometry. They showed in their work that LC-MS/MS gave more accurate and reliable results as compared to LC-UV and LC-MS [60]. Kau *et al* also developed a sensitive HPLC-UV and

microdialysis method to simultaneously determine the concentration of unbound ropivacaine in blood and brain obtained from rats [61]. Abdel-Rehim *et al* developed a new technique called microextraction in a packed syringe (MEPS) for preparation of samples in combination with liquid chromatography tandem mass spectrometry. They determined ropivacaine and its metabolites in human plasma using MEPS and LC-MS/MS [62]. Arvidsson and Eklund developed a method to determine only the free concentration of ropivacaine and bupivacaine in plasma by using an ultrafiltration technique. They used reversed phase and ion exchange columns in their study [63]. Koivisto *et al* compared the two techniques of ultrafiltration and microdialysis for the preparation of samples and then separated and determined the free concentration of ropivacaine in human plasma using packed capillary liquid chromatography. They reported that both the methods gave the same results i.e. the free concentration determined by both the methods was 6% [64]. Bergstrom and Markides again determined the free concentration of ropivacaine and its metabolites in spiked plasma by coupling of online microdialysis to packed capillary column liquid chromatography and tandem mass spectrometry [65]. Watson *et al* developed a selective and sensitive method for the determination for ropivacaine in plasma from neonates after epidural injections. The free and bound ropivacaine was separated by using a small scale equilibrium dialysis technique. The free drug concentration was then determined by solid phase extraction and liquid chromatography coupled with mass spectrometry [66]. Reif *et al* developed an HPLC method for determination of ropivacaine and its

metabolites [67]. Zou *et al* simultaneously determined the concentration of ropivacaine and antipyrine in perfusate samples by using HPLC-UV detection. The method was then applied to determine the transplacental transfer of ropivacaine performed through using an open model of perfused human placenta [68]. Tanaka *et al* also determined the three local anaesthetics ropivacaine, bupivacaine and mepivacaine by high performance liquid chromatography-UV in human serum and demonstrated the clinical and forensic applications of their method in the determination or identification of these three drugs [69]. Kawata *et al* developed a method based on reversed phase high performance liquid chromatography for the determination of ropivacaine, the method was then used to assess the concentration of ropivacaine in plasma of two volunteers who were given ropivacaine 0.5% w/v viscous preparation. They showed that the use of ropivacaine viscous was safe based on plasma concentrations of ropivacaine [70]. A study regarding the pharmacokinetic profile of two ropivacaine formulations was carried out by Montan *et al*. In this study 0.5 % w/v ropivacaine which was liposome encapsulated and 0.5% w/v ropivacaine in combination with 1:200,000 epinephrine were given to volunteers. Determination of ropivacaine was carried out by HPLC-UV for both of the formulations and it was found that there was no difference in any of the PK parameters. This indicated that a liposomal preparation could be a safer alternative to use with ropivacaine in place of a vasoconstrictor [71]. Salama and Wang determined the concentration of ropivacaine and bupivacaine in spiked human plasma as well as in authentic and pharmaceutical samples, the method was developed by using a time of

flight electrospray ionization mass spectrometer without the prior separation by liquid chromatography [72]. Wiedemann *et al* determined the concentration of the free and bound concentrations of ropivacaine in plasma after a long continuous use of epidural analgesia over 120 hours. The results suggested that the both the free and bound concentrations were within the safety limits and it could be safely used for continuous epidural analgesia [73]. Corso *et al* monitored free and bound levels of ropivacaine in a patient receiving ropivacaine infusions following bowel surgery [74]. The percentages of free ropivacaine were *ca* 1.5% throughout the 96 h post-surgery infusion and the lack of accumulation of free levels could be related to a 63% increase in the levels of AGP measured at 48 h post-surgery. A study of the PK of ropivacaine in neonates following the use of ropivacaine to carry out a single caudal block found that the levels of free drug ranged between 1 and 12% of total drug and there was a slight increase in the levels of AGP post-surgery [75]. In a similar study examining ropivacaine in neonates for both single shot and continuous infusion of ropivacaine the percentage of unbound drug were < 10% of the bound drug and AGP levels were found to increase post-surgery [76].

2.1.11 Aim of the study

The aim of the current study was to develop a method for measuring free and bound ropivacaine in patients undergoing THA and TKA at the Golden Jubilee hospital in Clydebank. The patients were given LIA postoperatively and there was some concern

about the possible build-up of toxic levels of free ropivacaine over time. In addition the aim was to measure levels of AGP in these samples.

Following from our previously developed equilibrium dialysis method in this study the rapid microscale equilibrium dialysis method recently described by Curran *et al* [77] was used and combined with analysis using hydrophilic interaction chromatography in combination with high resolution full scan mass spectrometry. In order to measure AGP a method previously developed at Strathclyde using HPLC was applied [78].

2.2 Experimental

2.2.1 Chemicals and materials

HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. Analar grade formic acid (98%) was obtained from BDH-Merck, UK. Sodium phosphate monobasic dihydrate, sodium phosphate dibasic, sodium chloride, α-1 acid glycoprotein and trifluoroacetic acid were purchased from Sigma-Aldrich, UK. The Thermo Scientific Pierce Rapid Equilibrium Dialysis (RED) units and base plate were purchased from Fisher Scientific, Leicestershire, UK. Ropivacaine hydrochloride monohydrate was obtained from the European Pharmacopoeia Laboratories, Strasbourg, France. Lidocaine hydrochloride monohydrate was obtained from Sigma Aldrich, UK.

2.2.2 Patient plasma samples

Blank plasma was provided by the Blood Transfusion Service (Gartnavel Hospital, Glasgow). Samples of plasma from patients were obtained from Dr Mike Gill from patients undergoing knee joint surgery at the Golden Jubilee Hospital in Clydebank. Full risk assessments were produced for the procedures and samples were obtained with informed patient consent and ethics committee approval. In this procedure a total of 200 mls of 0.2% w/v ropivacaine hydrochloride was injected into the anterior and posterior compartments of the knee and subcutaneously. An intra-articular catheter was left in the knee. Then the local anaesthetic administered as three 40 ml top-up doses via the intra-articular catheter in the first 24 hrs with two additional 'as needed' top-up boluses at least 20 minutes apart. Four mls of blood were taken for a baseline level of local anesthetic at the start of the procedure before the spinal anaesthetic. Then further samples of blood were taken at 5, 10, 15, 20, 25 and 30 minutes, 1 hour and 24 hours following local anaesthetic infiltration.

2.2.3 Preparation of different standards

Standard stock solutions of ropivacaine.HCl and lidocaine.HCl were prepared at 1 mg/ml in methanol.

2.2.4 Buffer preparation and equilibrium dialysis

First of all a buffer containing 100 mM sodium phosphate and 150 mM NaCl pH 7.40 was prepared by the following method [77]. A basic solution was made by dissolving 14.2 g/l Na_2HPO_4 and 8.77 g/l NaCl in deionized water. An acidic solution was made by

dissolving 15.6 g/l $NaH_2PO_4.2H_2O$ and 8.77 g/l NaCl in deionized water. The basic solution was then titrated with the acidic solution to pH 7.40. Immediately before dialysis plasma samples and buffer were temperature equilibrated at 37°C. The free and bound fractions of the drug were separated by equilibrium dialysis using RED (rapid equilibrium dialysis) device inserts (Fisher Scientific, Loughborough, UK) along with the required base plate. Each insert is composed of two side-by-side chambers which is separated by an O-ring-sealed vertical cylinder of dialysis membrane (MWCO ~8,000).The base plate is high grade reusable plate made of durable and chemically inert high-grade PTFE eliminating nonspecific binding. Each insert was filled with 300µl of patient or control plasma and 500µl of buffer respectively. Equilibration was then carried out for 6 hours at 37°C with shaking at 300 rpm in an Eppendorf Thermomixer.

2.2.5 Preparation of samples and extraction method

After six hours shaking the device was removed from the Thermomixer. Then 50µl was taken from the plasma and buffer compartments and transferred into an Eppendorf tube and 50µl of IS (lidocaine 1µg/ml in water) solution and 10µl of 0.1% v/v aqueous formic acid were added to each tube. Then 50µl of blank plasma was added to the buffer sample and 50µl of buffer was added to the plasma sample [77] and the samples were vortexed. Then 340µl ACN was added to both the plasma and buffer samples and the samples were vortexed for 2 minutes and centrifuged at 9000 rpm for 10 minutes. After centrifuging the supernatant layer was taken directly for LCMS analysis.

2.2.6 LC-MS analysis

Measurement of standards and samples was carried out on a Surveyor HPLC system combined with an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). The column used was a ZIC-HILIC column (150 × 4.6mm, 5µm Hichrom, Reading, UK). Mobile phase A consisted of 0.1% v/v formic acid in water and Mobile phase B consisted of 0.1% v/v formic acid in ACN. The flow rate was 300 µl/min. The ESI interface was operated in a positive ion mode with a spray voltage of 4.5 kV. The temperature of the ion transfer capillary was 275°C and the flow rates of the sheath and auxiliary gases were 50 and 17 arbitrary units respectively. The full scan range was 75 to 1200 m/z. The data was recorded using Xcalibur 2.1.0 software (Thermo Fisher Scientific). Mass calibration was performed for both ESI polarities before the analysis using the standard Thermo Calmix solution and the signals at 83.0604 m/z (2xACN+H) was selected as lock masses for positive ion mode during each analytical run. The following extracted ion ranges ($[M+H]^+$) were monitored: m/z 275.21-275.22 for ropivacaine and m/z 235.175-235.185 for lidocaine.

2.2.7 Calibration curve

Diluted stock solutions of ropivacaine were prepared at a concentration of 10µg/ml and 1µg/ml in water and stored at 4°C and a diluted stock solution of the lidocaine internal standard was prepared at 10µg/ml. Then 1 ml aliquots of plasma were spiked with 0 µg, 0.01µg, 0.03µg, 0.05µg, 0.1µg, 0.2µg, 0.5 µg, 1 µg, 1.5 µg, 2 µg of ropivacaine and 1µg of lidocaine internal standard. Then these samples were processed in the

same way as patient samples but without a dialysis step. A linear range was thus established over the range of 0.01–2.0 μg/ml.

2.2.8 Precision and accuracy of the method for determination of bound drug

The precision and accuracy of the method was determined by repeat preparation (x5) of the 0.03 and 1.5 μg spiked samples of plasma. These samples were processed in the same way as the patient samples. The recovery from the protein precipitation procedure was determined by treating six replicates of plasma (for high level) and buffer (for low level) each without the internal standard. The internal standard was then added to both the extracts from plasma and buffer in the reconstitution step.

An ionization suppression test was carried out by mixing 0.4 ml of blank plasma + 0.4 ml of buffer with 3.2 ml of acetonitrile and the sample was centrifuged and the supernatant was removed. The supernatant (500μl) was spiked with 50μl of ropivacaine (0.5 μg/ml); similarly 500μl of acetonitrile was also spiked with 50μl of ropivacaine (0.5 μg/ml). The samples were then analysed.

2.2.9 Precision of the method for the determination of free drug

The precision for the determination of free drug was also checked by spiking 5ml of blank plasma with 50μl of a solution of ropivacaine (0.1 mg/ml) in water. Then 5 aliquots from this spiked plasma were treated using the RED method and the levels of the free drug in dialysate were determined.

2.2.10 Determination of the precision of analysis of unbound drug in samples

Repeat (n=5) determination of bound and unbound drug in a selected patient sample was carried out.

2.2.11 Determination of binding capacity of patient samples

An aliquot of patient plasma (1ml) was spiked with ropivacaine to produce a concentration of ropivacaine at 1µg/ml above the original concentration.

2.2.12 Determination of AGP levels in patient plasma

The method for determination of AGP is described in detail in chapter 3.

2.3 Results

2.3.1 Calibration

The HILIC method proved convenient for the analysis of ropivacaine since when using a protein crash with organic solvent there is no need to remove the organic solvent since trapping in HILIC mode is most efficient with a high concentration of organic solvent in the sample solution. Figure 2.4 shows the chromatograms obtained for lidocaine and ropivacaine extracted from plasma and dialysate and then analysed on a ZICHILIC column interfaced to an LTQ Orbitrap mass spectrometer.

The method was calibrated over the range 0.01-2.0 µg/ml by using spiked blank plasma. Since the blank was found to be clear of ropivacaine the calibration curve was forced through zero otherwise the intercept had a disproportionate impact on the

lower concentration samples. The equation of the line was y = 0.886x ± 4.2% (n=2) and R^2 was 0.997 ± 0.15% (n=2).

2.3.2 Accuracy and precision

The values obtained for the accuracy and precision for the analysis of plasma spiked at 0.03 μg/ml and 1.5 μg/ml were 93.2% ± 2.8% and 95.4% ± 1.5% respectively (n=5). Repeat analysis of a patient sample for free and bound drug gave the following values for levels of ropivacaine: bound 1.63 μg/ml ± 1.48%, unbound 0.0671 μg/ml ±1.68% (n=5). Thus the microscale equilibrium dialysis process worked with a good degree of precision and was suitable for the analysis of free and bound ropivacaine in plasma samples from patients. The full data for the levels of free and bound drug in patients is reported in a clinical journal [79] with comment of the significance from clinical point of view. The full set for the determination of free and bound ropivacaine are shown in Appendix 1. In total about 760 samples were analysed including a re-run of the hip replacement samples since in the first set it was found that the infusion pump delivering the drug infusion was malfunctioning. Having found a good degree of linearity for the full calibration curves the level of drug in each batch of patient samples was determined by using a single point calibration prepared by spiking ropivacaine and lidocaine at 1 μg/ml into blank plasma. The mean ratio obtained across 25 such spikings on 25 different days over a period of seven months was 0.939 ± 9.9% indicating that the variation in slope based on a one point spiking was within acceptable limits.

2.3.3 Recoveries, ion Suppression and robustness

In the recovery test the recovery of 0.5 μg/ml spikings of ropivacaine into plasma was 91.7% ± 8.9% (n=6) and for 0.05 μg/ml spikings the recovery was 90.1±3.7% (n=6). In the ion suppression test the ratio of the signal for a 0.05 μg/ml spike of ropivacaine into supernatant from a plasma crash compared with acetonitrile was 1.007±5.6% (n=2). The robustness of the methodology was tested by spiking ropivacaine into a patient sample already containing ropivacaine in order to further assess ion suppression effects and recovery. In the sample selected the initial levels of drug were 0.389 μg/ml bound and 0.00433 μg/ml (1.12%) unbound. The level of bound drug after spiking at 1 μg/ml was 1.38 μg/ml ± 0.68% (n=3) and the level of unbound drug was 0.0263 μg/ml (1.92%) ± 1.6% (n=3) thus the plasma proteins offered strong buffering against an increase in the level of free drug.

2.3.4 Determination of AGP levels in patient plasma samples

The AGP levels were determined for knee arthoplasty samples and also for the first set of hip arthoplasty samples. The results obtained from the AGP determinations are shown in Appendix II. The chromatograms showing AGP in spiked and unspiked plasma is reported in chapter 3.

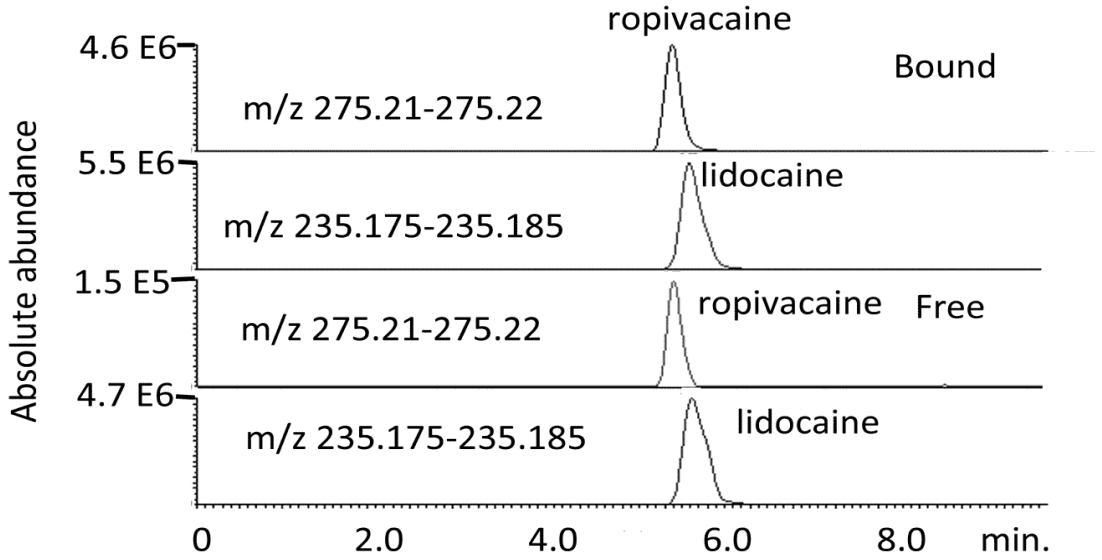


Figure 2.4 Extracted ion chromatograms for bound and free ropivacaine and lidocaine internal standard (1 µg/ml).

2.4 Some additional observations on the metabolism of ropivacaine

Since the analysis was carried out in full-scan mode on the Orbitrap a lot of data was collected on the metabolism of ropivacaine. This was not the primary focus of the work but it is worth reporting some of the observations. Figure 2.5 shows the structures of some of the main potential metabolites of ropivacaine detected by high resolution LC-MS. All of these metabolites apart from M1 can be seen in plasma at 5 minutes. However, looking more closely at the data the peak for M2 was present in the baseline pre-dose samples. Examining the protocol for anaesthesia it was observed that the patients were given gabapentin pre-operatively and by coincidence gabapentin has exactly the same mass and formula as M2. Hydrolysis of the amide bond in ropivacaine

as a route of metabolism in ropivacaine has not been reported but has been found to occur in earlier versions of these types of anaesthetics such as prilocaine [43].

Thus it appears that this is not a route of metabolism for ropivacaine. The M3 and M4 have been described before but the literature does not mention the formation of the glucuronide M5. Such phase II metabolites may have an important effect on the duration of action of ropivacaine. It appears that the glucuronide is the main metabolite at 1 hour although the dealkylated metabolite M3 is highest by 24 hours. Figures 2.6-2.8 show extracted ion traces for the potential metabolites shown in figure 2.5 in a patient at 5 minutes, 1 hour and 24 hours.

MS/MS study was also done for ropivacaine and the the results of MS fragmentation give a main peak at m/z 126 which confirmed as a fragment of ropivacaine. Figures 2.9 and 2.10 show the MS/MS data of ropivacaine showing the main fragment at m/z 126 in 5 minute and 24 hour samples.

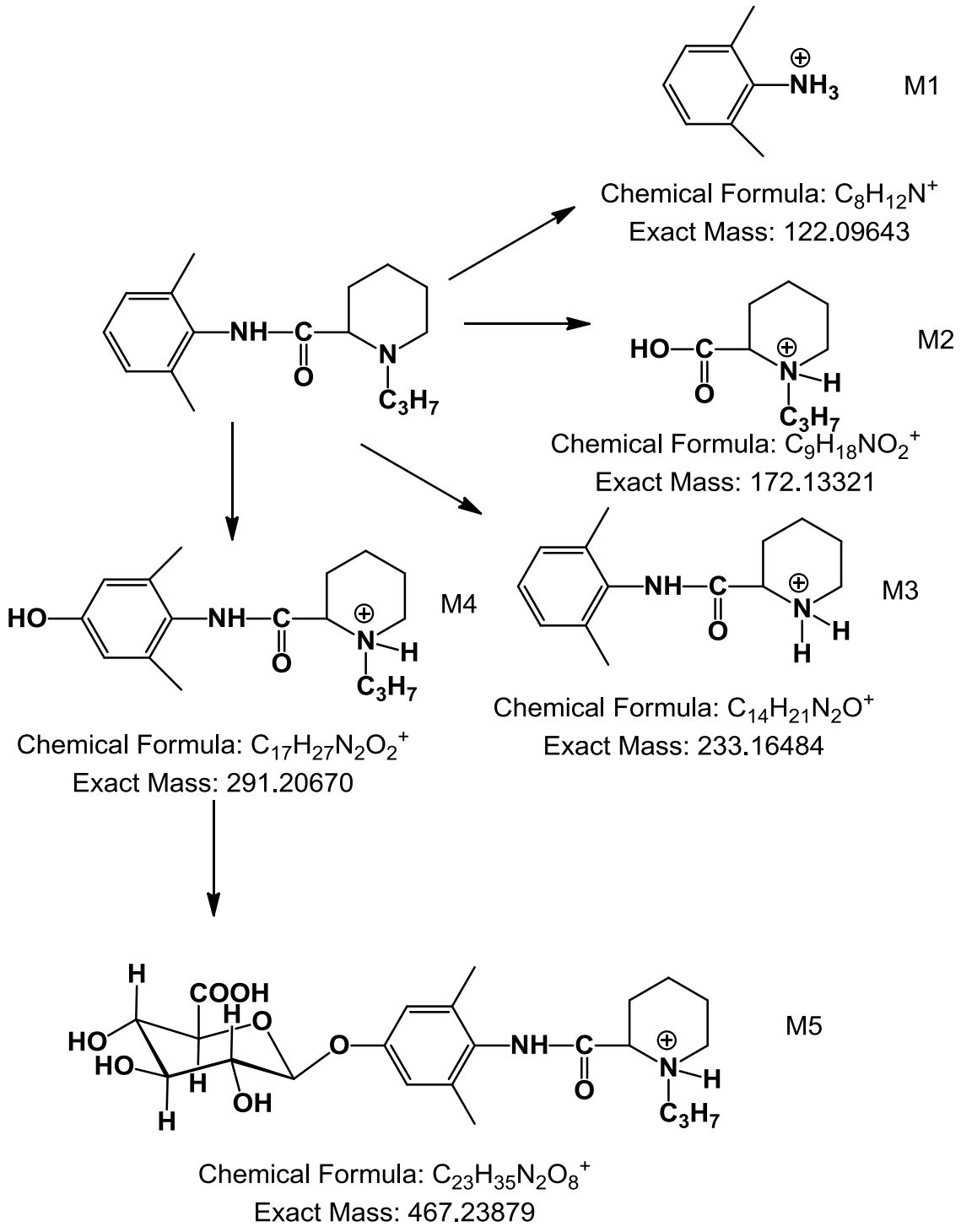


Figure 2.5 Possible metabolites of ropivacaine

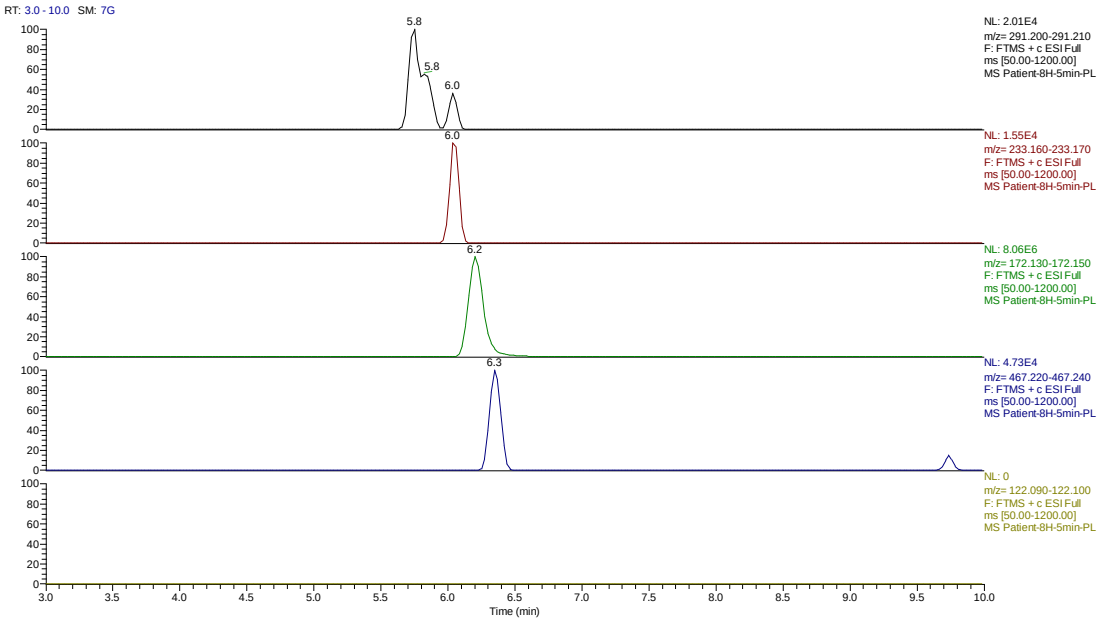


Figure 2.6 Metabolites of ropivacaine in plasma at 5 minutes.

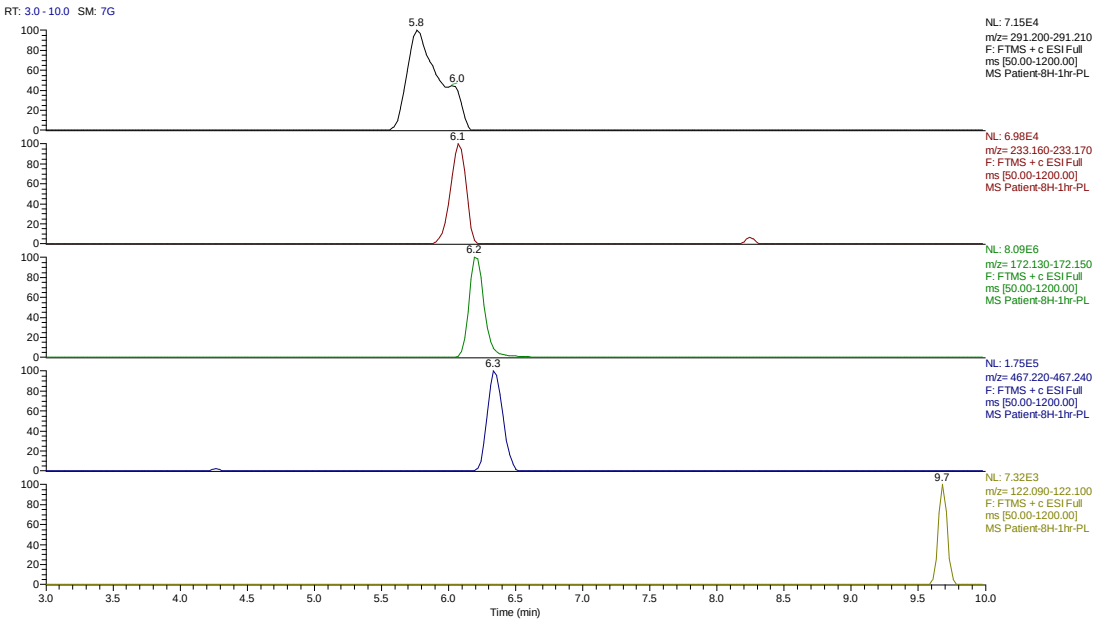


Figure 2.7 Metabolites of ropivacaine in plasma at 1 hour

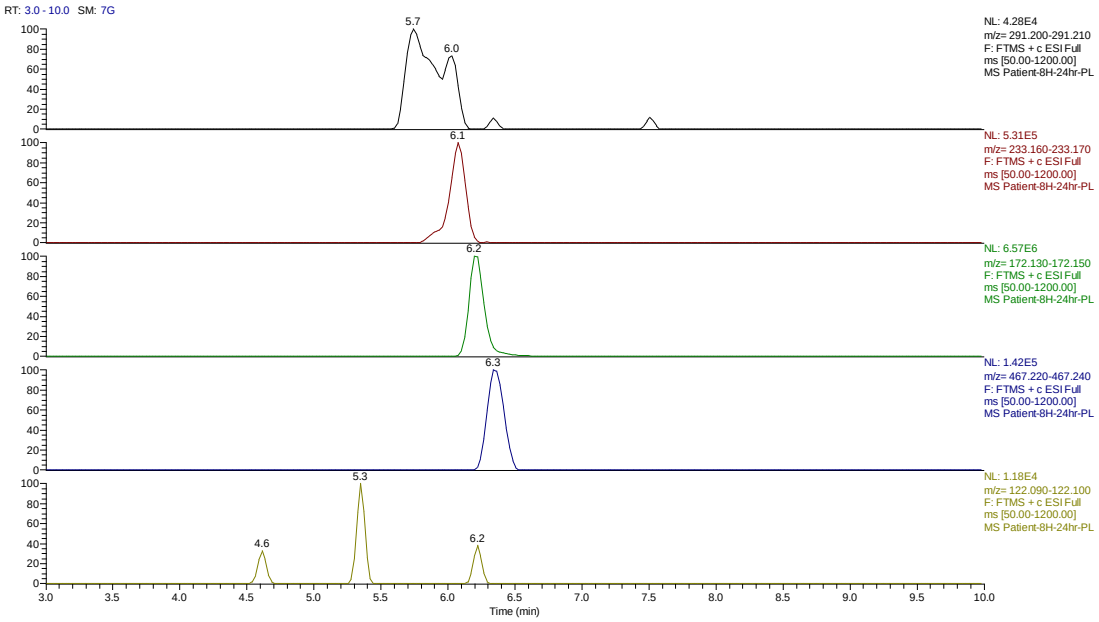


Figure 2.8 Metabolites of ropivacaine in plasma at 24 hours

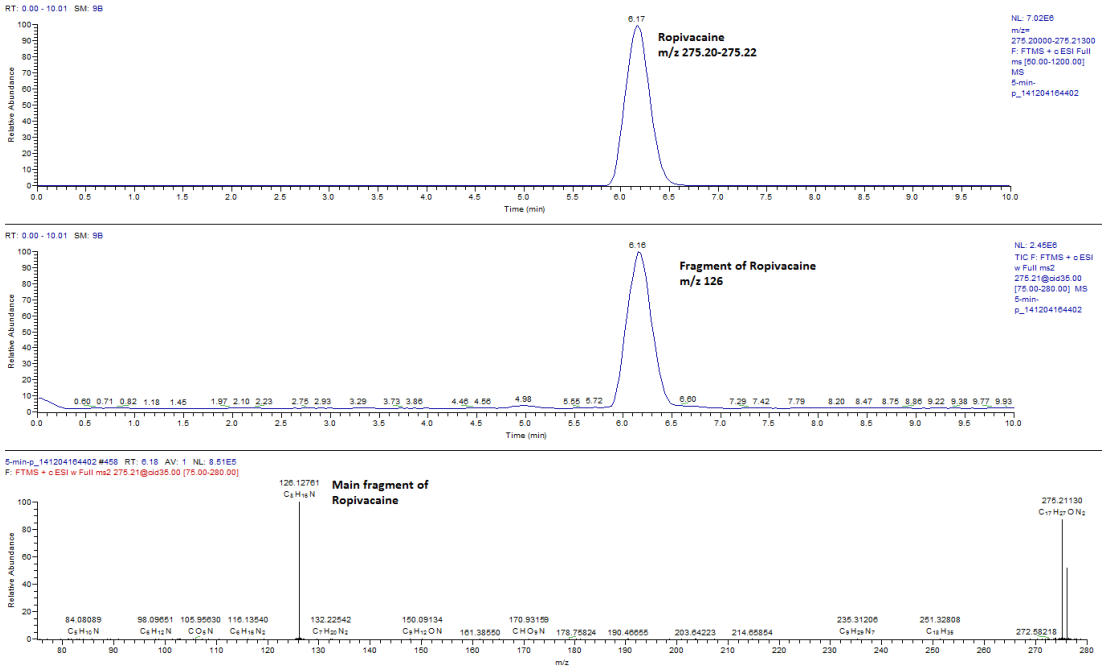


Figure 2.9 MS/MS of ropivacaine showing the main fragment at m/z 126 in 5 minutes

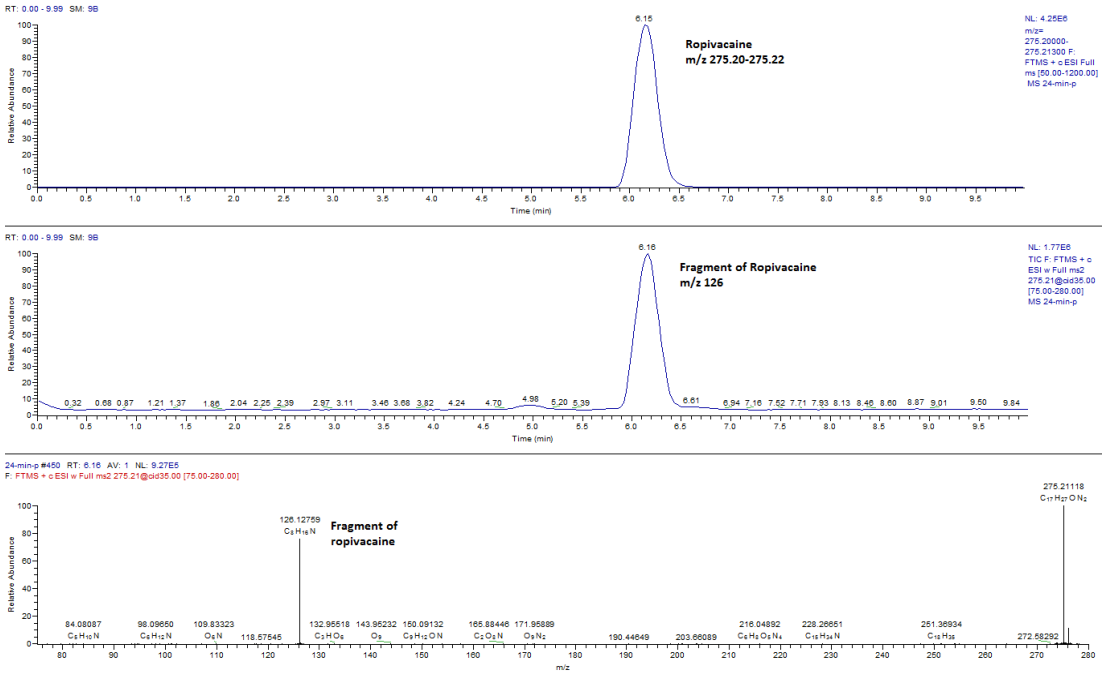


Figure 2.10 MS/MS of ropivacaine showing the main fragment at m/z 126 in 24 hours

2.5 Conclusion

A robust and precise method was developed for the determination of free and bound ropivacaine in plasma and published in Talanta [80]. The RED devices proved convenient to use in comparison with a method which had been developed previously for the determination of levels of unbound drug. The HILIC method works ideally with a protein crash method since there is no requirement to reduce the high levels of organic solvent which can cause peak distortion when reversed phase methods are used. The method has been applied to the analysis of free and bound drug in 30 patients undergoing knee surgery and 20 patients undergoing hip surgery. The results indicate that the levels of free drug remain below levels which are regarded as safe. The clinical data has been published [79] and is already well cited and thus has made an important contribution to practice. The levels of AGP which were determined in each sample and will be correlated with the observed levels of free drug in a pharmacokinetics paper.

Chapter 3

3 High Performance Liquid Chromatography Determination of α 1-Acid Glycoprotein in Human Plasma

3.1 Protein binding

Drug binding to different proteins in plasma may have significant importance in drug therapy because drug protein binding serves as a depot for the drug and has an influence on the pharmacokinetic, pharmacodynamic parameters and metabolism of the compound [81]. The pharmacological action of the drug is only due to the unbound drug and not the total drug present in the plasma [82, 83]. Thus the effect of plasma protein binding on the pharmacokinetic and pharmacodynamic parameters needs careful attention. Many cases were reported by different authors where a small change in plasma protein binding resulted in clinically vital changes in the pharmacokinetic parameters of the drug. In contrast a limited number of articles are available regarding the effects of protein binding on the pharmacodynamics of a drug and a number of different factors need to be addressed which include mechanism of action of drug, affinity of drug for the target site and protein, number of binding positions of drug on the target site and protein, location and concentration of protein at the target site [84].

Drug protein binding is a well explained phenomenon in humans. According to the regulatory procedures and guidelines in most countries of the world it is obligatory for the pharmaceutical industry to carry out different studies required for a new drug which include determination of various *in vitro* and *in vivo* pharmacokinetic parameters. The information obtained from these pharmacokinetic parameters should then be used to develop a safe dosage regimen with the aim to provide an effective target drug concentration to the patient. Overall the purpose is to achieve optimal efficacy with a minimal chance of toxicity. Generally researchers have a good knowledge regarding the role of these pharmacokinetic parameters in developing a drug dose; however the adjustment of drug doses in the case of variation in pharmacokinetic parameters needs more knowledge and attention [85].

The relationship between pharmacokinetics and pharmacodynamics has been explained by the free drug theory (FDT). According to the free drug theory, that without any energy dependent process and if the equilibrium state is achieved, the concentration of free drug in the plasma or extracellular space is equal to the concentration of free drug in the tissue or intracellular space and only the unbound portion of the drug in the tissue is able to bind to receptors to produce the pharmacological response. Therefore the free or unbound concentration of the drug in plasma is pharmacologically relevant to the free concentration of the drug in the tissue. The concentrations of total drug in the plasma and in tissues may be different from each other to a large extent but for those drugs having high rate of permeation the

concentration of free drug in the tissue equals the concentration of drug in extracellular space or in the plasma. Similarly plasma protein binding information is very important in drug-drug interactions related to metabolism in order to determine the free concentration of the drug that causes inhibition of drug metabolizing enzymes which might ultimately result in a toxic effect [86].

3.1.1 Unbound fraction/unbound concentration

It is the concentration of the drug which is not bound to plasma proteins and only this concentration of the drug produces the pharmacological effect. This unbound concentration of the drug varies during the dosing interval and needs to be accurately and correctly explained to determine the effect of the drug [85].

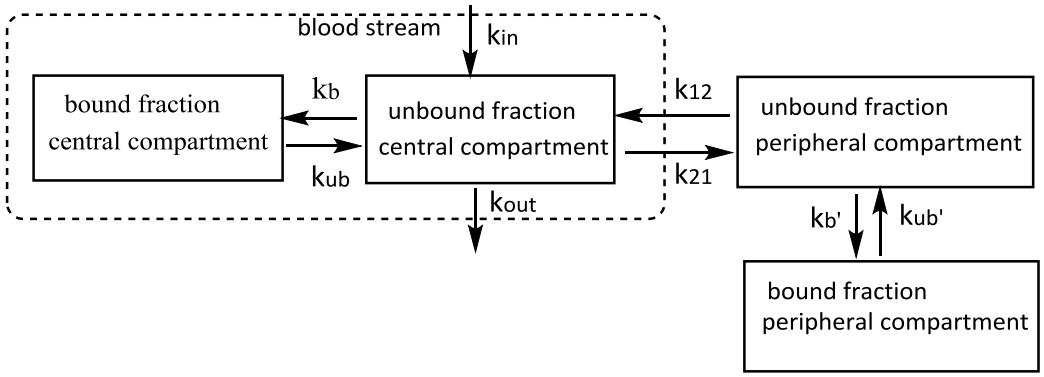


Figure 3.1 Shows the equilibrium between central and peripheral compartment

3.1.2 Effect of protein binding variation on pharmacokinetics

Drug binding to plasma proteins has a very vital role in determining the diffusion rate of the drug between extracellular and intracellular space, therefore any change in this diffusion affects pharmacokinetic parameters including volume of distribution and rate

of clearance of the drug [86]. Figure 3.1 represents a two compartment model showing bound and unbound concentration in plasma and tissue. From the figure it is clear that the bound concentration of the drug and distributed drug in the tissue influences the concentration of unbound drug, both of which also serve as depot for the unbound drug in the plasma (central compartment). Only the unbound concentration of the drug is excreted from the body. Re-equilibration is established between the bound fraction and the distributed drug to maintain the concentration of unbound drug. Sometimes in rare situations the concentration of unbound drug decreases rapidly before the equilibrium is established again. This occurs if the distribution rate or dissociation from plasma proteins is slower as compared to drug clearance. The key point with regard to the equilibrium is that concentration of the unbound drug is going to decrease in the later stages of the dosing interval because excretion of the drug from the body decreases both the unbound concentration and reservoirs which act to maintain its concentration. Figure 3.1 also explains how drug clearance and volume of distribution is increased when the protein binding decreases [85]. Generally in case of highly protein bound drugs, a little change in protein binding is responsible for significant changes in the unbound concentration of drug, thereby increasing the clearance and volume of distribution [85, 86].

Both volume of distribution and clearance of total drug are affected by protein binding, according to equation

$$Vd = (fu / fuT)\, V_T + V_P \qquad (1)$$

Where;

fu = unbound fraction of drug in the plasma,

fuT = unbound fraction of drug in the tissue,

V_T = Tissue volume,

V_P = Plasma volume.

The equation (1) shows that Vd is directly proportional to fu, it means that if the unbound concentration of the drug increases as in the case of certain diseases, the volume of distribution also increases.

Clearance (CL) can be determined by

$$CL = Q(f_u \cdot CL_{int}) / (Q + (f_u \cdot CL_{int})) \quad (2)$$

From the equation above it is clear that the clearance of the drug depends on (Q) blood flow to the elimination organ, (f_u) unbound concentration of the drug and (CL_{int}) intrinsic clearance which varies by enzymatic activity or tubular secretory activity of kidney.

There is a marked increase in the clearance of drugs which are mainly removed by glomerular filtration, because of increased renal blood flow associated with high intrinsic clearance and/or increased in the concentration of unbound drug.

Equations 1 and 2 show that both the volume of distribution and clearance of drugs increases if there is decrease in plasma protein drug binding [85].

3.1.3 Causes of changes in protein binding

Many factors have been reported in the literature that affects drug protein binding. In diseases like cancer, liver and kidney diseases, in patients with burn injuries and in diabetic patients, protein binding alteration is more common. The effectiveness of a drug in these cases depends on whether the drug is acidic in nature or a basic drug and also depends on whether the drug binds to albumin to which acidic drugs normally bind or to α1-acid glycoprotein to which basic drugs bind. Plasma protein binding is also reported to be affected by temperature and pH. However, it is noteworthy that the changes produced by these factors in the unbound concentration of the drug are not likely to occur to the same extent or to affect the success and efficacy of the treatment to the same extent. Hinderling and Hartmann worked in detail in this area and proposed no significant variations in clinical outcomes with pH changes except for fentanyl and lidocaine. Drug displacement from protein binding sites by another strongly binding drug will also have a significant affect on the concentration of the displaced drug and dose adjustment should be taken into consideration in the use of such drugs [85].

According to literature the following conditions are necessary to be present in patients if changes in protein binding are to be considered to be related to significant differences in clinical outcomes.

These are:

i) Drugs having a high tendency of protein binding

ii) High clearance rate drugs

iii) Drugs where dose titrations have no significant effect. Dose titration is the adjustment of the dose to achieve the desired effect. So for drugs where dose titration produces an effect, changes in plasma protein binding is unlikely to produce a significant effect unless the drug has a very narrow therapeutic window. For example in vasopressor therapy the effect of the drug dosing can be determined by monitoring the blood pressure, and dosing can be adjusted according to the blood pressure. However in the case of antibacterial drugs the direct pharmacological effect is difficult to measure unless by determination of the concentration of the drug in the blood. So in this case the drug is not titrated to produce an effect [85].

3.2 Acute phase proteins

Acute phase proteins are produced during the acute phase reaction in the body to various stimuli and have a role in the defence response shown by the body. Their levels also give an idea about the progression of the disease or inflammatory response via monitoring their concentrations in the blood [87]. The changes in the plasma concentration of these proteins lead to altered drug binding and ultimately affect the pharmacokinetic and pharmacodynamic parameters for the drug [88]. Among the acute phase proteins α1-acid glycoprotein is the most important and major protein [88, 89].

3.2.1 Alpha-1-acid glycoprotein

α1-Acid glycoprotein was described for the first time by Karl Schmid and Richard J. Winzler and their colleagues in 1950 and who considered it as an unusual protein because of its low p*I* value which ranges from 2.8-3.8 and has a comparatively highly content of carbohydrate (45% w/w) [78, 87, 90, 91]. α1- Acid glycoprotein was considered to have the highest carbohydrate content of any protein until the characterisation of galactoglycoprotein in 1980 which has a 76% of carbohydrate content [92, 93, 94].

α1- Acid glycoprotein (AGP) or (AAG) also called orosomucoid (ORM), is the main protein responsible for binding basic drugs [78, 88, 93, 95, 96, 97, 98, 99, 100]. α1- Acid glycoprotein and human serum albumin are the most abundant proteins present in the plasma. Human serum albumin is the most concentrated protein in the plasma at a concentration of 4g/100ml while the quantity of α1- Acid glycoprotein in the plasma ranges between 40 and 100 mg/100ml [78, 101]. Although AGP concentration is far lower than that of human serum albumin, AGP is considered as an important and significant protein for drug binding having important clinical effects [98]. Also AGP is reported to have a role in drug-drug interactions due to the displacement of drugs and other endogenous molecules from their binding sites resulting in significant clinical implications [98]. Approximately 300 drugs are known to bind to AGP [99].

Human AGP is acidic in nature and its molecular weight is about 40-43 kDa [78, 88, 93] or 41,000 g/mol [92]. AGP has a single polypeptide chain of 183 amino acids and five N-

linked carbohydrate chains [98]. The carbohydrate part contributes about 40% of the molecular weight of AGP and is responsible for its high solubility and acidic properties [96]. AGP is present in the serum in different forms because of the differences in the sequence of amino acid and also because of the type and number of carbohydrate moieties that are attached to the polypeptide chain [92].

The synthesis of AGP mostly occurs in the liver and then it is secreted by hepatocytes [88] but there is also an evidence of extra hepatic synthesis [97].

The concentration of AGP in serum increases in various conditions like inflammation, after surgery, due to burn injury, cancer and various other conditions [90, 94, 97, 98, 99, 102]. There is also variation in the levels of AGP among the healthy individuals depending on the age, sex and hormonal variations [99].

Many articles have been published on AGP since 1950 but the exact range of biological functions of AGP is still unclear [88, 93], however the immunomodulating effects and the property of binding basic drugs has been described many times by different authors [93] and these properties have been demonstrated to be mainly dependent on the carbohydrate content of the protein [88]. AGP is also responsible for transportation of both endogenous and exogenous substances to their target sites [87, 99].

3.3 Experimental

Human α1-acid glycoprotein, trifluoroacetic acid and perchloric acid (70%) were purchased from Sigma Aldrich (UK). HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific, UK. Blank plasma was provided by the Blood Transfusion Service (Gartnavel Hospital, Glasgow). Samples of plasma from patients were obtained from Dr Mike Gill from patients undergoing hip or knee joint surgery at the Golden Jubilee Hospital in Clydebank. The samples were stored at −20^{o}C until analysis.

3.3.1 HPLC analysis

The high performance liquid chromatographic system used in the analysis consisted of a Spectrasystem P2000 gradient pump, a Spectrasystem AS3000 autosampler (equipped with a Type 7010-150 Rheodyne injection valve (20µl loop)) and a Spectrasystem UV 1000 detector. The column used in the analysis was a PLRP-S 4000 °A column (50mm×4.6mm I.D., 5µm particle size, Agilent technologies). The mobile phase consisted of 1ml TFA/L of water (solvent A) and 1ml TFA/L of acetonitrile (Solvent B). The gradient used was as follows: 0 min 18% B, 15 min 60% B, 17 min 60% B followed by column re-equilibration for 7 min before the next injection. The flow rate of the mobile phase was 1 ml/min. The detection wavelength was set to 220 nm. The acquired data was processed by using ChromQuest software [78].

3.3.2 Standards preparation

Stock solution of AGP was prepared at a concentration of 2 mg/ml in water. Different calibration solutions were then prepared from the stock solution by diluting it in 0.5 M perchloric acid. Different standards at a concentration of 6.25, 12.5, 25, 50 and 100 mg/100 ml were prepared and injected directly into the chromatographic system.

3.3.3 Sample preparation

The concentration of AGP in the plasma was determined by the following method: to 50µl of patient or control plasma, 100µl of 0.5 M of perchloric acid was added and were vortexed for 20 s in Eppendorf tube to mix it thoroughly. The acidified plasma was then centrifuged at 9000 rpm for 5 minutes at room temperature. The supernatant was then transferred to the insert in the HPLC vial for analysis. Both the extracted standards and samples were stored at 4^{o}C before they were chromatographed.

The concentration of AGP was then calculated from the linear correlation of AGP concentration versus values of peak areas obtained from the perchloric acid extract and expressed as mg/ 100 ml of plasma.

3.4 Results and discussion

The margin of therapeutic safety for many drugs like anaesthetics should be considered in older age because of the ageing process occurs in the body which may result in toxicity of the drugs. There are very few data published regarding the binding of local

anaesthetics to plasma proteins. This study was performed to determine the level of AGP in the plasma of elderly hip and knee joint surgery patients.

The level of AGP in the plasma increased during the postoperative study period which supported the view that the level of AGP increases during and following surgical procedures. Similar results were shown in a previous study [78] which determined the concentration of AGP in neonates and also by two other studies [100] and [103].

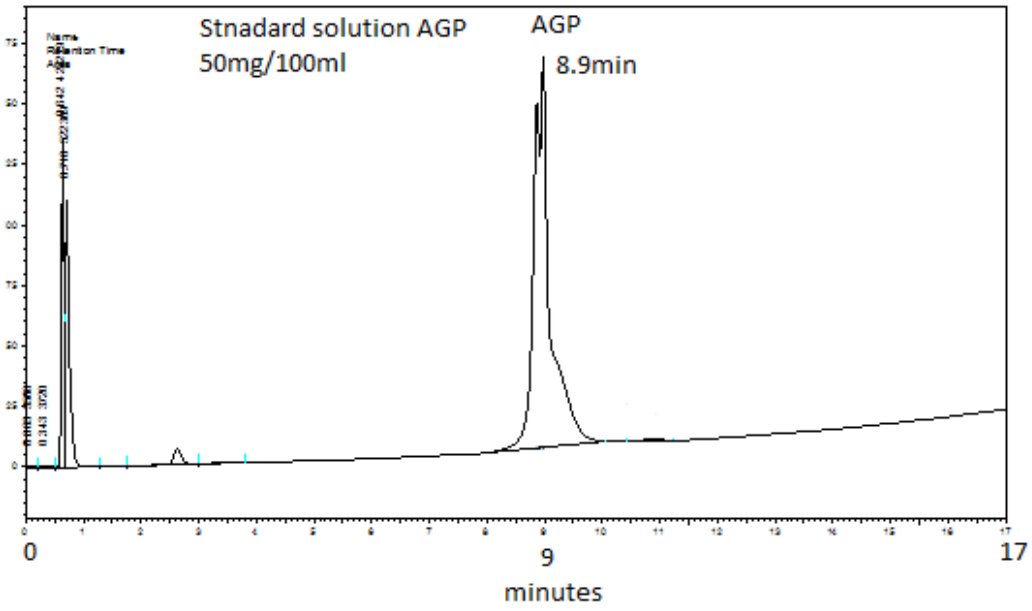


Figure 3.2 Chromatogram of an unextracted aqueous standard containing 50 mg/100 ml of AGP (conditions as in section 3.3.1)

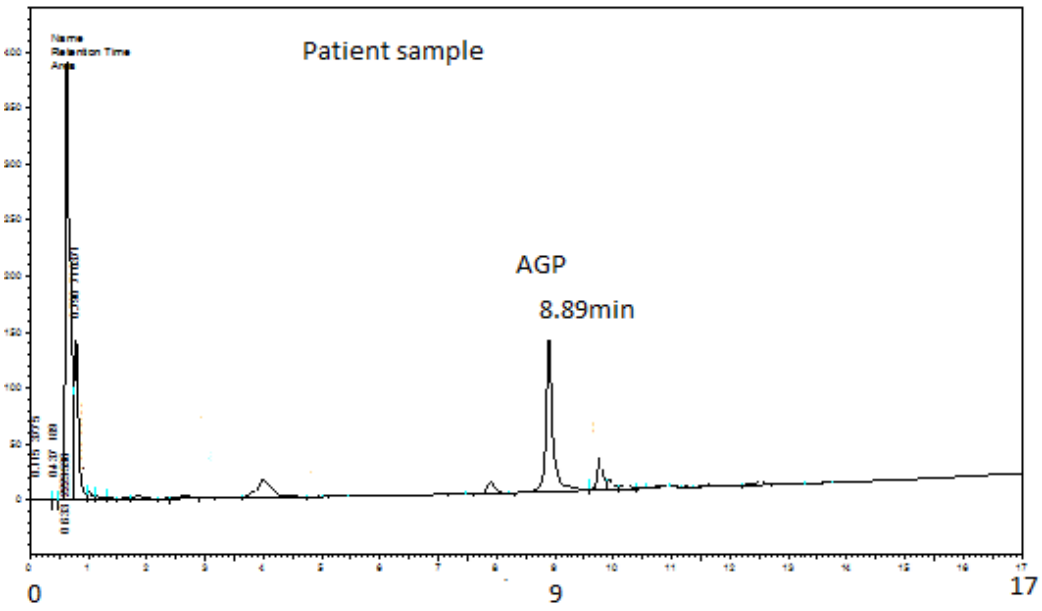


Figure 3.3 Chromatogram of AGP extracted from 50 µl of the plasma (conditions as in 3.3.1)

The normal mean value for the concentration of AGP in the plasma is 77 mg/100ml [100]. However there is great variation of this value among individuals. The results we obtained in our analysis gave concentrations which were generally below those specified by previous publications.

The AGP concentrations were analysed in the plasma of 20 hip joint surgery patients and 20 knee joint surgery patients. These samples were taken at 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 1 hr, 4 hr, 24 hr. Figure 3.2 shows a typical chromatogram of the AGP standard injected directly into the chromatographic system and Figure 3.3 shows a chromatogram of the AGP in patient plasma following removal of the rest of the protein with 0.5 M perchloric acid. The peak for AGP was obtained between 8.8 and 8.9 minutes. We follow a method developed previously [78], the method works

very well for the determination of AGP and there is minimal interference from other serum proteins e.g. HSA which is precipitated out almost completely by the perchloric acid; AGP remains in solution because of its high water solubility which is due to its 40% carbohydrate content. The calibration curve obtained by plotting the areas of the AGP Peaks versus concentration of AGP on different days was linear Figure 3.4. The data obtained for calibration curve on three different days and the average of this data is shown in table 3-1 and table 3-2. The calibration curve covered the concentration range from 6 mg/100 ml to 100 mg/ 100 ml which also covered the concentration of AGP in the patient plasma. Having established linearity a single point standard of 50 mg/100ml was run every day with the patient samples. The results for the AGP concentrations in patients receiving a continuous infusion of a local anaesthetic ropivacaine following total knee or hip arthroplasty are presented in appendix 2.

Table 3.1 Results for calibration curve obtained at three different days

Conc (mg/100ml)	1st day	2nd day	3rd day	Median	Std D	%RSD
6.25	415958	344383	394228	394228	29962.38	7.60027
12.5	719000	699826	759672	719000	24951.95	3.47037
25	1461052	1515656	1672708	1515656	89718.81	5.91947
50	3156967	3077156	3504651	3156967	185593.9	5.87887
100	6211756	6816230	7250593	6816230	425994.6	6.24971

Table 3.2 Averages of the three different calibration curve values

Concentration(mg/100ml)	Peak area(average)
6.25	394228
12.5	719000
25	1515656
50	3156967
100	6816230

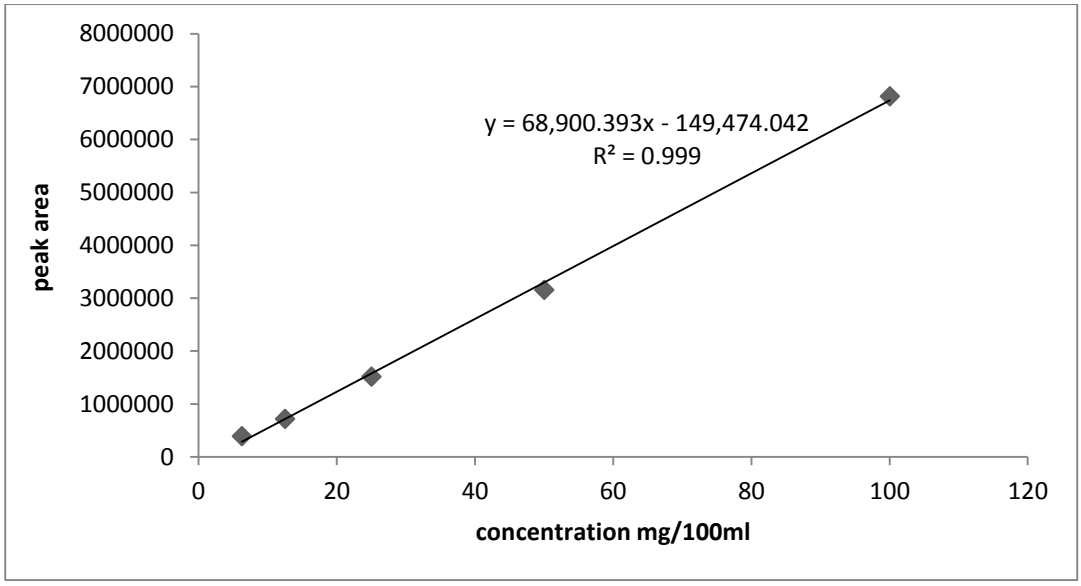
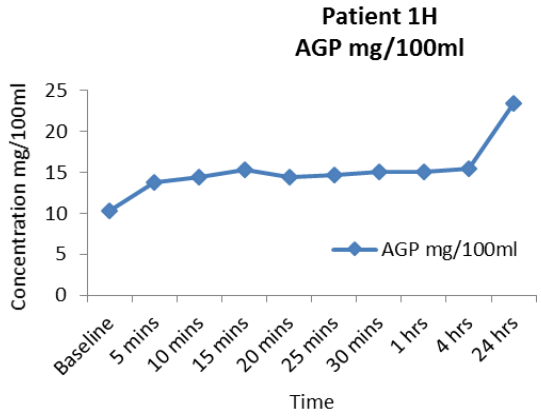


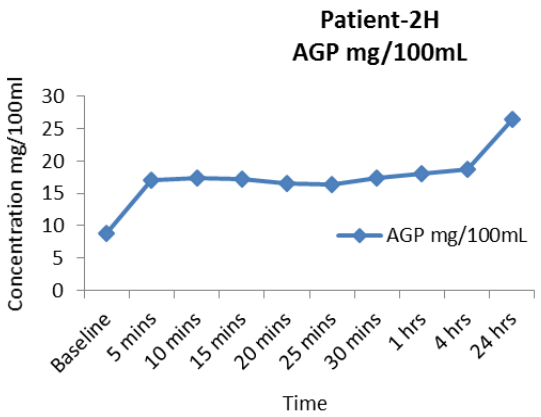
Figure 3.4 Calibration curve used for AGP determination

As can be seen from the results attached in the appendix 2 and the graphs below (fig 3.5), nearly all the patients have AGP levels which are higher after 4 hours and 24 hours as compared to the samples taken at early times. This increase in the AGP level is directly related to plasma protein binding which increases in the samples taken after 24 hours (data reported in the first chapter). The increased plasma protein binding was associated qualitatively with the unchanged free concentration of the drug. Thus this

stress related increase in the concentration of AGP resulted in increased of plasma protein binding of the drug (ropivacaine) which in turn keeps the free concentration of ropivacaine stable during the postoperative period.

In the analysis of AGP mostly there is interference from other plasma proteins when determining it, this is mainly from human serum albumin which has similar characteristics to AGP and thus affecting the resolution of AGP. Therefore perchloric acid was used in the method to ensure the complete removal of HSA compared to the use of methanol-chloroform for extraction which failed to remove the HSA [78]. Also AGP is very stable when present in acidic solution and remains soluble in water because of its extensive glycosylation. As there were many problems associated with reverse phase columns when used for the separation of proteins, the separation of AGP was carried on a short PLRP-S 4000 Å from Agilent technologies. These columns are designed specifically for the analysis of proteins. This column has an advantage of wide pore polymer based support and consists of rigid and macroporous polystyrene/divinylbenzene spherical particles, which are resistant to extremes of the pH range and can therefore be used directly for analysis of an acidic perchloric extract. The column was very robust for this analysis and its performance did not deteriorate during the analysis of *ca* 700 samples.


Patient 1H
AGP mg/100ml
Concentration mg/100ml
25
20
15
10
5
0
AGP mg/100ml
Baseline
5 mins
10 mins
15 mins
20 mins
25 mins
30 mins
1 hrs
4 hrs
24 hrs
Time


Patient-2H
AGP mg/100mL
Concentration mg/100ml
30
25
20
15
10
5
0
AGP mg/100mL
Baseline
5 mins
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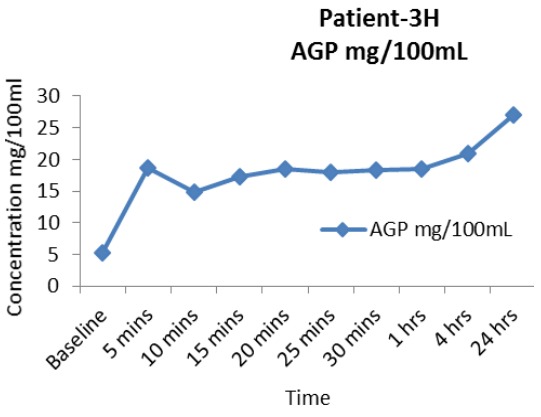
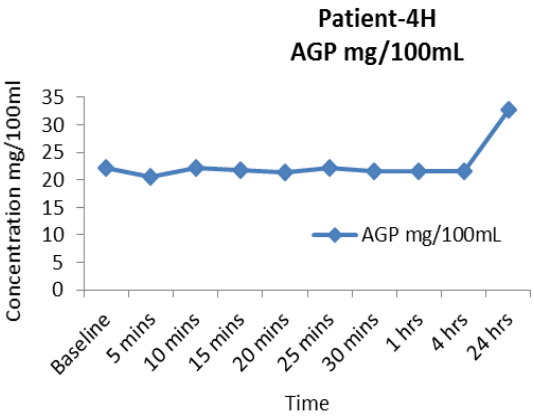
25 mins
30 mins
1 hrs
4 hrs
24 hrs
Time


Patient-3H
AGP mg/100mL
Concentration mg/100ml
30
25
20
15
10
5
0
AGP mg/100mL
Baseline
5 mins
10 mins
15 mins
20 mins
25 mins
30 mins
1 hrs
4 hrs
24 hrs
Time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AGP mg/100mL
Concentration mg/100ml
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30
25
20
15
10
5
0
AGP mg/100mL
Baseline
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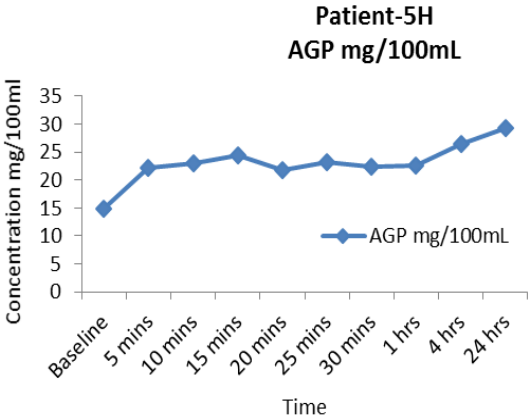
20 mins
25 mins
30 mins
1 hrs
4 hrs
24 hrs
Time


Patient-5H
AGP mg/100mL
Concentration mg/100ml
35
30
25
20
15
10
5
0
AGP mg/100mL
Baseline
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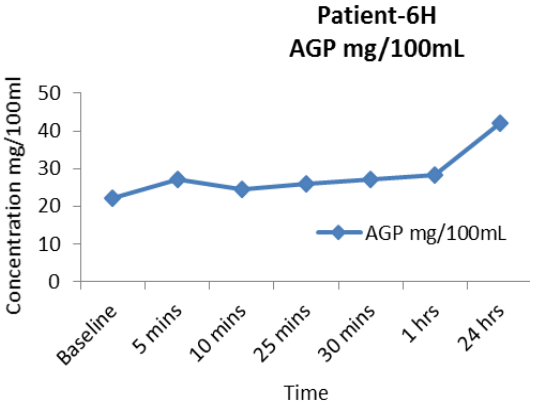
20 mins
25 mins
30 mins
1 hrs
4 hrs
24 hrs
Time


Patient-6H
AGP mg/100mL
Concentration mg/100ml
50
40
30
20
10
0
AGP mg/100mL
Baseline
5 mins
10 mins
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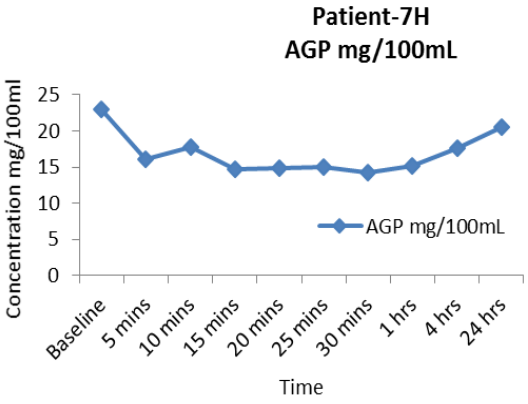
30 mins
1 hrs
24 hrs
Time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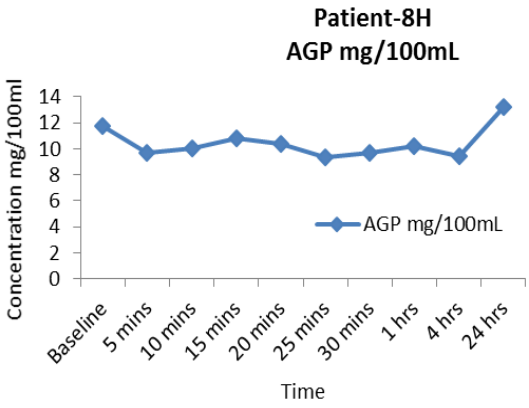
AGP mg/100mL
Concentration mg/100ml
25
20
15
10
5
0
AGP mg/100mL
Baseline
5 mins
10 mins
15 mins
20 mins
25 mins
30 mins
1 hrs
4 hrs
24 hrs
Time


Patient-8H
AGP mg/100mL
Concentration mg/100ml
14
12
10
8
6
4
2
0
AGP mg/100mL
Baseline
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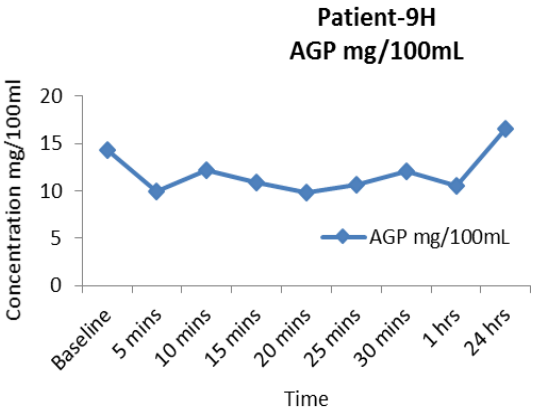
20 mins
25 mins
30 mins
1 hrs
4 hrs
24 hrs
Time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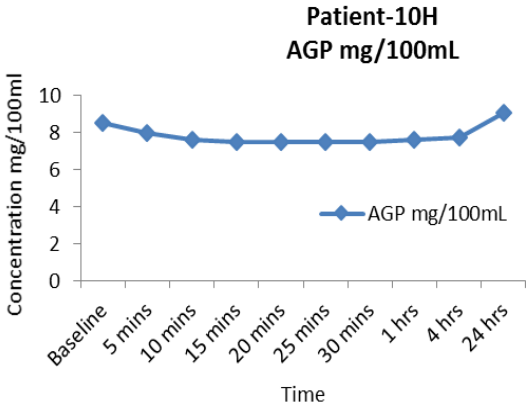
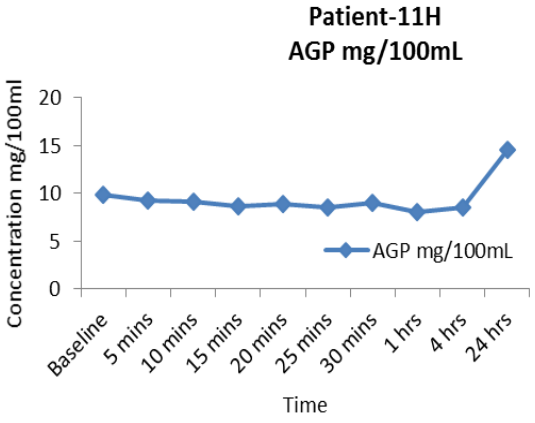
AGP mg/100mL
Concentration mg/100ml
20
15
10
5
0
AGP mg/100mL
Baseline
5 mins
10 mins
15 mins
20 mins
25 mins
30 mins
1 hrs
24 hrs
Time


Patient-10H
AGP mg/100mL
Concentration mg/100ml
10
8
6
4
2
0
AGP mg/100mL
Baseline
5 mins
10 mins
15 mins
20 mins
25 mins
30 mins
1 hrs
4 hrs
24 hrs
Time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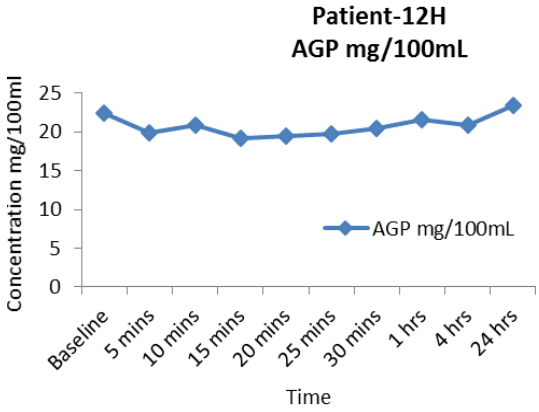
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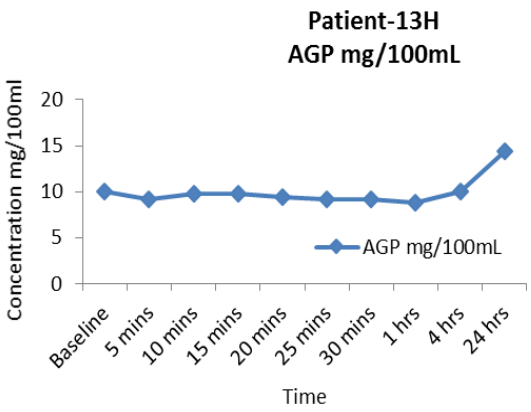
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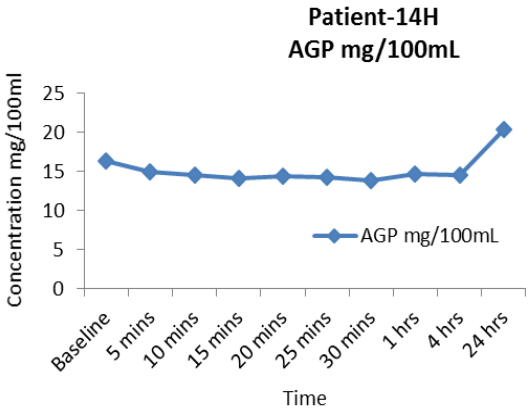
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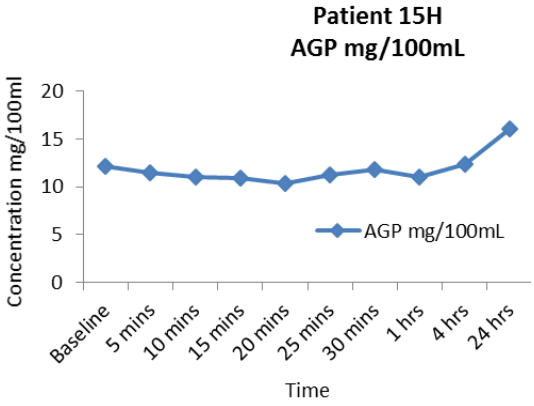
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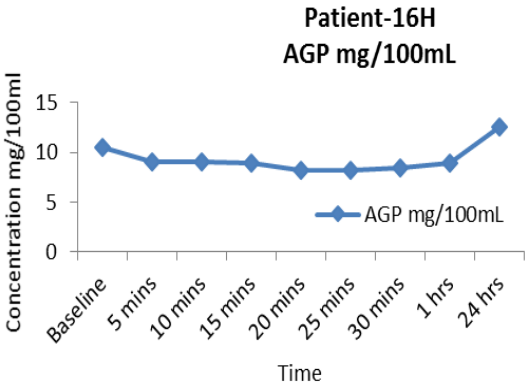
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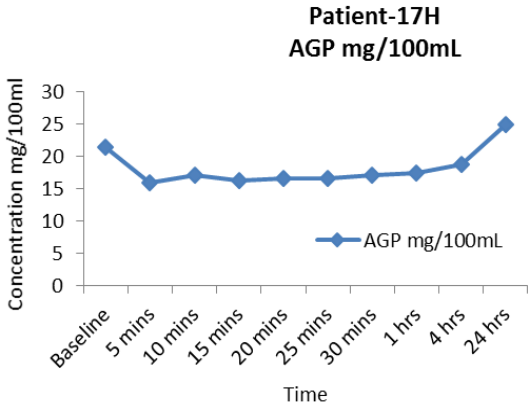
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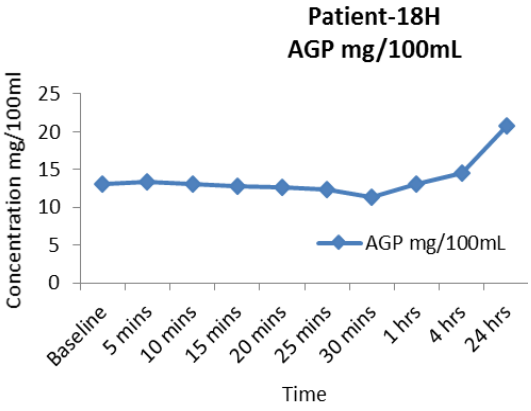
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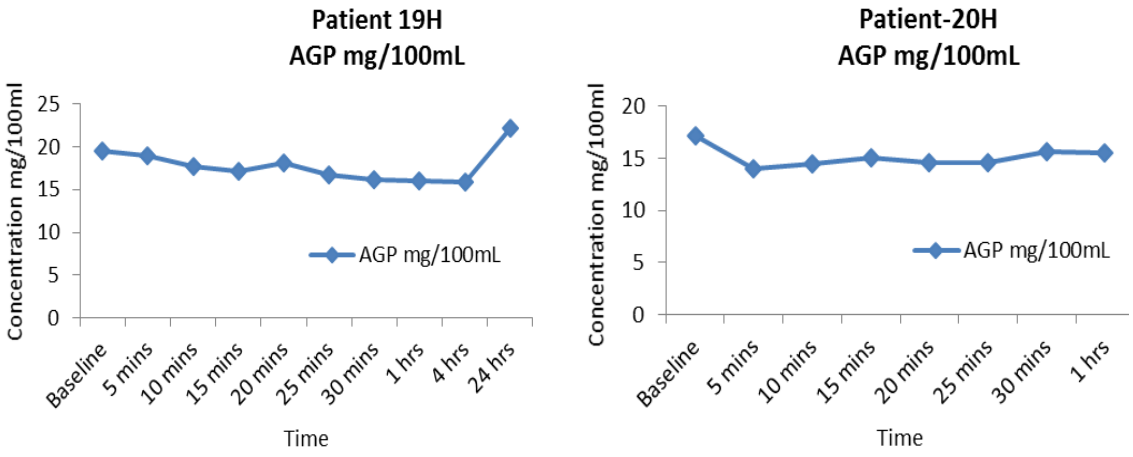


Figure 3.5 Graphical presentation of AGP concentration determined in the plasma of 20 hip surgery patients at different time interval.

Chapter 4

4 Determination of Estrone, Estradiol and their metabolites by high performance liquid chromatography coupled with mass spectrometry

4.1 Introduction

Estrogens are female sex hormones which have an important role in development and maintenance of the female reproductive system and are responsible for controlling the secondary gender characteristics of females [104, 105]. These hormones also have a role in bone health and in the neuroprotective process [106]. The menstrual cycle and pregnancy are also regulated and maintained by estrogens. Estradiol is considered to be the most active of the natural estrogens; it is produced by the ovaries and is mainly bound to plasma proteins [104].

Estrogens are considered as small lipophilic molecules and derived from their precursor molecule cholesterol which serves as a backbone for the synthesis of all steroids in different parts in the body including gonads, adrenal glands, and placenta and to a small extent in adipose tissue and liver. Synthesis of Progesterone, 17β-estradiol and testosterone results from the oxidative activity of two enzymes cytochrome P450 and hydroxysteroid dehydrogenase. Steroid hormones synthesis is regulated partly by the hypothalamic-pituitary axis. Leuteinising hormone (LH) is synthesized and secreted by

the anterior pituitary and is responsible for promoting the synthesis of estrogens and testosterone in the gonads [107].

Cholesterol for the synthesis of estrogens can be obtained from three sources: it can be derived from the cell membrane; obtained from acetate or from high density lipoprotein or low density lipoprotein in the circulation. Cholesterol is metabolised to pregnenolone by the activity of CYP enzyme systems and steroidogenic acute regulatory proteins. Pregnenolone is then converted to dehydroepiandrostenedione (DHEA) by the catalytic activity of CYP enzymes. DHEA is then metabolised to androstenedione with the help of an enzyme 3β hydroxysteroid dehydrogenase (3β-HSD) which is further metabolised to testosterone by the catalytic activity of 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3) which is intercovertable to androstenedione by an enzyme called 17β-HSD2. Androstenedione can be converted to 16α-hydroxyandrostenedione by the activity of CYP2C11. The three steroids androstenedione, testosterone and 16α-hydroxyandrostenedione by oxidative metabolism and in the presence of NADPH give rise to the active estrogens including estrone, estradiol, estriol and 16α-estriol.

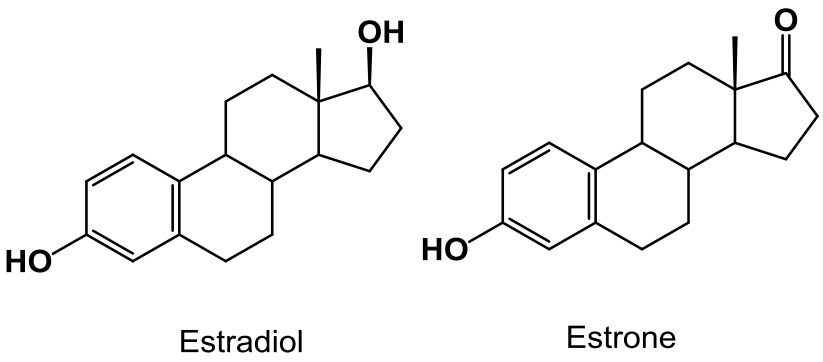


Figure 4.1 The structures of estrone and estradiol

Estrogens have three main subtypes which are derivatives of 17β-E2 estradiol (17β-E2), estrone (E1) and estriol (E3). The main circulating estrogen in premenopausal women is 17β-E2 and the concentration of this varies during the menstrual cycle. The concentration is normally about 100pg/ml or ~0.37nM while during the ovulation phase it reaches to around 600pg/ml or ~2.2nM. There is a significant decrease in the concentration of 17β-E2 after the menopause. The exogenous sources of estrogens and estrogen like compounds include contraceptives, dietary sources (phytoestrogens), estrogen replacement therapy and various chemical products such as plasticisers. Vascular cells also have the ability to convert locally testosterone or androstenedione to estradiol and estrone respectively [107].

4.1.1 The biosynthesis of estrogens

The biosynthesis of naturally occurring estrogens, which are C18 steroids, mainly occurs from cholesterol in the body. Cholesterol in the body binds to lipoprotein receptors and is then taken up by steroidogenic cells and this carries it to the site of steroid biosynthesis. Carrier proteins and the cytoskeleton facilitate this inward movement of

cholesterol. Different steroids are then produced from cholesterol by cleavage of the 27 carbon chain to form 18 carbon compounds (figure 4.2). The rate limiting step in the production of steroids is the transport of cholesterol to the inside of mitochondria where the enzyme cytochrome P450 is present that activates the cleavage of the cholesterol side chain. The last step in the production of estrogens is called aromatization. This reaction is catalyzed by a complex of enzymes present in the endoplasmic reticulum called P450 aromatase monooxygenase enzymes which function as demethylases. Estrone and estradiol are produced from their precursors as a result of three successive hydroxylation steps [108].

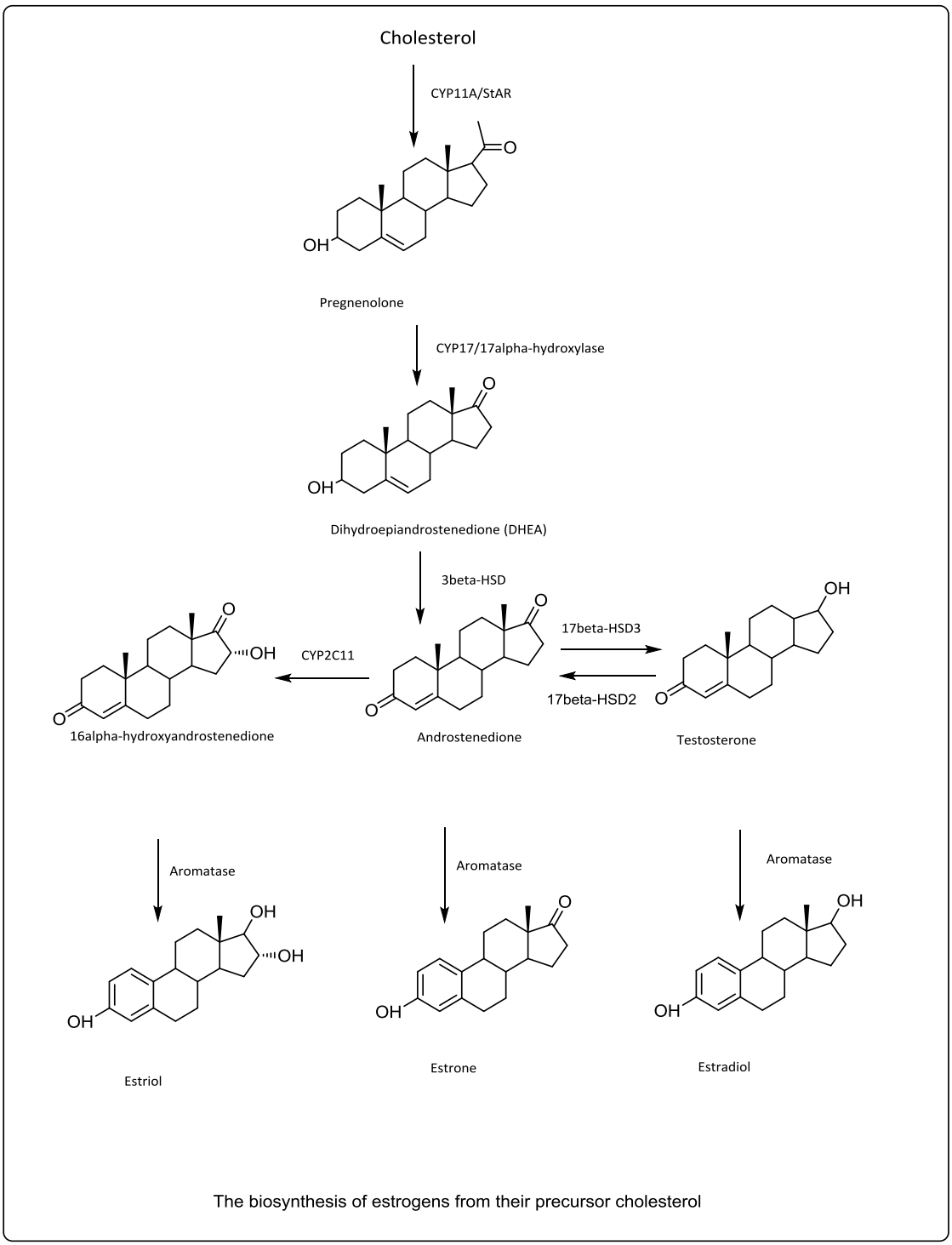


Figure 4.2 The biosynthesis of estrogens from their precursor cholesterol

4.1.2 Metabolism

The elimination of estrogens from the body occurs in the form of inactive or less active water soluble metabolites in urine and/or in faeces [109]. Estrogens are metabolised mainly in the body by: oxidative metabolism (hydroxylation); glucuronidation (conjugative metabolism) and sulfation and/or *O*-methylation. The estrogen conjugates are then excreted in the bile or urine. Cytochrome P450 is the primary enzyme responsible for catalyzing the NADPH dependent oxidation of estrogens to their hydroxylated metabolites. Metabolism of estrogens by hydroxylation is followed by methylation which results in the formation of catechols and methoxylated estrogens. Metabolism of estrogens by hydroxylation produces 2-hydroxyestrogens, 4-hydroxyestrogens and 16α-hydroxyestrogens. Methoxylated estrogen metabolites are produced from methylation of 2-hydroxyestrogens and 4-hydroxyestrogens by an enzyme called catechol *O*-methyltransferase [108].

Estradiol binds in a reversible manner mainly to β-globulin in the blood which functions as regulator of the concentration of free circulating steroids and helps in the transport towards its target sites. The steroids reach their target sites via their binding to β globulin which has a receptor in the plasma membrane of the cell thus resulting in either co-internalisation or the release of steroid at the target site. Steroids to a small extent also bind to albumin in a nonsaturable manner; only the free unbound portion of the steroid is considered to be biologically active [107, 108].

4.1.3 Sources of estrogens

The main sources of estrogens in premenopausal women are the theca and granulose cell ovaries. Ovaries produce estradiol, the circulating hormones acting on the distal target tissues. However, estrogens are also produced in extragonadal sites in postmenopausal women as ovaries stop the production of estradiol after menopause. They are also produced in extragonadal sites in men. As a number of extragondal sites produce estradiol in postmenopausal women they may act locally on the sites that produce them and are not only considered as endocrine factors. The sites that produce estradiol include vascular endothelium, the smooth muscle cells of aorta, different parts of the brain and adipose tissues including breast, osteoblasts and chondrocytes of bone [110].

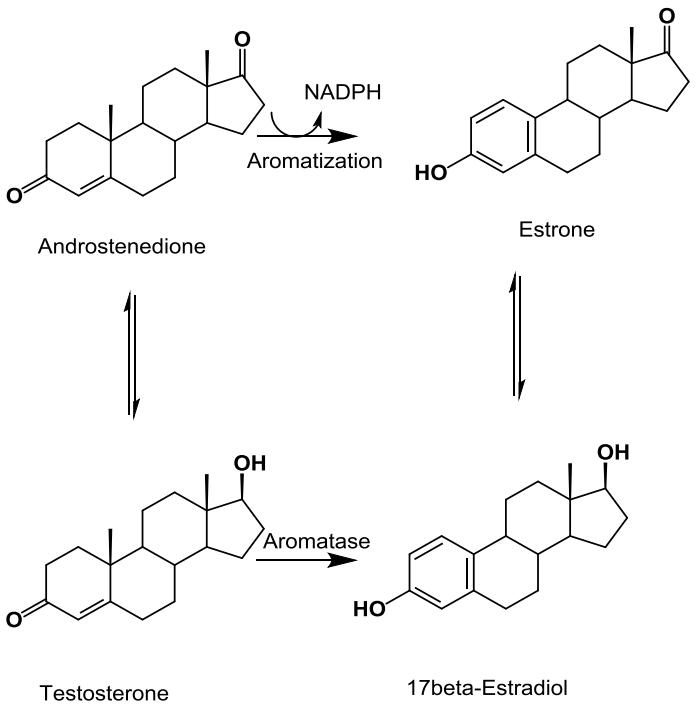


Figure 4.3 Aromatization of estrone and estradiol

4.1.4 Functions of estrogens

Estrogens mainly bind to two receptors to produce their actions and these receptors are known as ERα and ERβ [111]. Estrogens have many functions during the reproductive age of women and function as endocrine signalling molecules produced by the granulosa cells in the ovaries. The physiological role of estrogens in females includes:

- the growth of secondary sexual characters,
- regulation of the secretion of gonadotropin which promotes ovulation,
- tissue preparation for the response to progesterone,
- bone mass protection and maintenance,

- regulation of the biosynthesis of lipoproteins and insulin responsiveness,
- prevention of urogenital atrophy
- Continuation of cognitive functions.

The main function of estrogens in men is to help in the fusion of the epiphyses and maintain and regulation of bone mass in young age men [112].

4.1.5 Aromatization

The aromatase enzyme is present in the endoplasmic reticulum and is responsible for the biosynthesis of estrogens from their precursor steroids (figure 4.3). The main sites of aromatase expression are different between women and men. In premenopausal women the granulose cells of ovaries are the main sites of aromatase expression whereas in pregnant and postmenopausal women these sites are placental syncytiotrophoblasts and adipose and skin fibroblasts respectively. Both in men and postmenopausal women the principal mechanism of estrogen formation is the aromatization of the precursor C19 steroids which occurs in peripheral tissues i.e. adipose tissue and skin. In males testicular steroidogenesis produces about 15% of the estrogens present in the circulation. The amount of estrogens formed as a result of aromatase expression in different areas like ovaries, placenta, skin and adipose tissue is sufficient for them to be quantified in the blood. In all these areas of steroid production, the steroid precursors which act as a substrate for aromatase are produced by different cell types from those cells which express the aromatase. These cells which produce the precursor, 16α-hydroxyandrostenedione, may be either distal or adjacent

to the cells expressing aromatase. The precursor cells are distal in case of skin and adipose tissue. The main precursor cells in the case of placenta are the adrenal cells and liver of fetus. Other examples are the granulose cells of ovaries where precursor is obtained from the adjacent theca cells and the brain where the aromatase expressing tissues are neurons and astrocytes. It has been shown the activity of aromatase is stimulated by testosterone. The sites of aromatase expression in human bones are osteoblasts, chondrocytes, and adipose fibroblasts [112].

4.1.6 Derivatisation for mass spectrometric analysis

Determination of very small quantities of compounds in biological matrices needs an accurate and sensitive method. Liquid chromatography coupled with mass spectrometry has become an important analytical tool because of its sensitivity, accuracy and selectivity. However, the detection is extremely difficult in LC-MS if the compound under investigation is difficult to ionize in the ion source resulting in low detection sensitivity. The performance can be increased by advancing the instrument design, through better sampling procedures and enhanced chromatographic performance.

Chemical derivatisation has been found to be a powerful technique for enhancing the detection of compounds under investigation by making them more ionisable in the LC-MS system. A number of derivatisation reagents and protocols have been developed for the determination of steroids. The technique of chemical derivatisation for LC-MS analysis started in the 1980s and after that there has been a continuous growth and

progress in the technique for the analysis of a variety of compounds that were difficult to analyse without chemical derivatisation. The aim of the derivatisation is to introduce a moiety into the structure resulting in the modification of the structure of the target compound changing their physical and chemical properties so that they are more ionisable in the LC-MS system. The advantages of chemical derivatisation for LC-MS analysis (figure 4.4) reported in different reviews are:

- Improvement in the selectivity and separation of the target analyte.
- Increasing the ionization efficiency in the mass spectrometry system for detection.
- Enhancement in structure elucidation.
- Help in isomer separation.
- Elimination of endogenous interference.

Derivatisation is a specific type of chemical reaction that needs a functional group present on the target compound and reacting group in the derivatisation reagent for the derivatisation procedure to be completed. The prerequisites for an effective derivatisation based analysis in LC-MS is that the derivatization reaction should not be very long, should be effective and selective and the resultant product should be stable. The derivatisation reagent is commonly selected on the basis of functional group present in the structure of the target compound along with the research purposes after chemical derivatisation. Following derivatisation [113] the physical and chemical characteristics of the target compound may alter affecting the ionization efficiency,

retention time in liquid chromatography, stability and solubility of the compound. So the chemical structure of the target compound defines the strategy for the selection of best the derivatisation reagent for the research purpose.

During the derivatisation procedure the derivatising reagent reacts with the functional group of the target compound. This functional group may be hydroxyl, carboxyl, carbonyl, amine or thiol. On the basis of these functional groups derivatizing reagents can be classified into different groups. So it is very important in LC-MS analysis to select the proper derivatization reagent for the best and most reliable results [113].

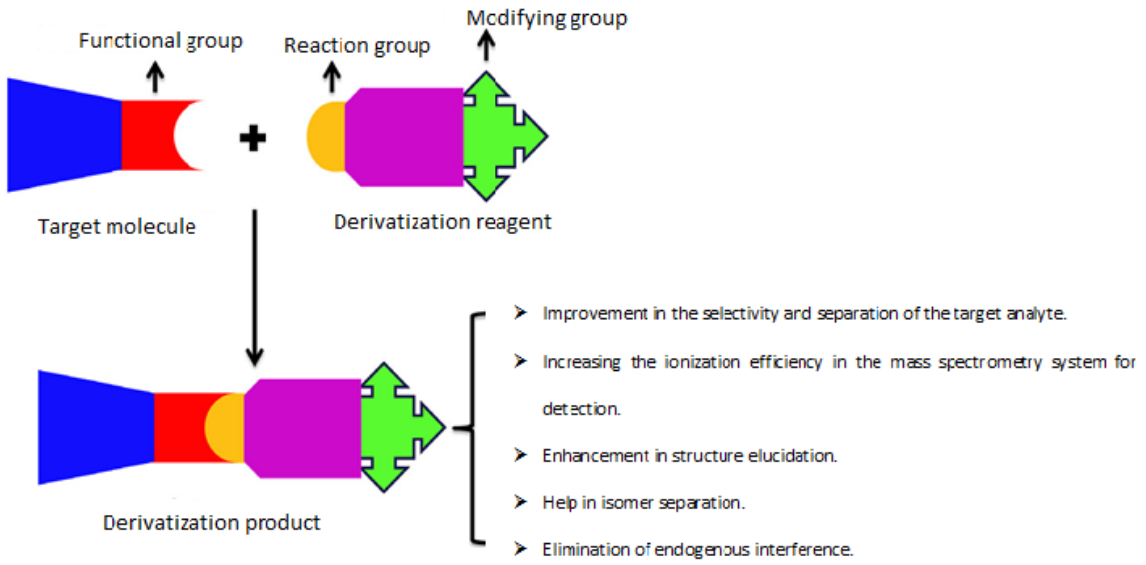


Figure 4.4 Reaction of a derivatisation reagent with the functional group of a target compound. Figure reproduced from [113]

4.1.7 Derivatization of steroids

Steroid hormones possess a very strong biological activity in the body even in very low concentrations (in picograms/ml) and both natural and synthetic steroids are used in the body for therapeutic purposes. So a very well defined method is needed to determine the concentration of both endogenous and exogenous steroids in the body in order to determine the nature of any disease present and thus be helpful in the diagnosis of the disease and in its treatment. Determining these steroids in the body is a challenging task because of their structural similarities and their low concentrations in the body. A variety of techniques have been used for the determination of estrogens by different scientists. Liquid chromatography mass spectrometry is the most recent technique employed for the determination of steroids because of its selectivity and versatility. However, the ionization capability of most of the steroids is not very good in mass spectrometry systems and is thus not satisfactory for trace amounts of steroids. Chemical derivatisation is now commonly used to overcome this problem. In chemical derivatisation a non-ionisable compound is converted to a moiety that is highly ionisable in the mass spectrometer and thus easily detected by the system. Different derivatisation reagents have been synthesized and are available depending on the functional groups of the target steroids [114, 115, 116, 117].

There are many factors that can influence the production of ions and signal intensity in the mass spectrometer [118]. ESI has now become the most important technique for the production of gas phase ions from a variety of compounds but the detection

sensitivity of ESI is extremely poor for neutral steroids. The best detection of steroids can be obtained if the analyte is either in ionic form or can be readily ionized in solution [114]. For this purpose scientists have produced more ionisable derivatives by modifying the steroid structure in order to enhance the detection of non-ionisable compounds. As all estrogenic compounds have a phenolic group in the structure, this functional group can be employed to attach the API sensitive moieties (figure 4.5). In most of the derivatisation techniques used for analysis in ESI an ionisable group or permanently charged group is attached to the analyte in order to enhance their detection [114, 118].

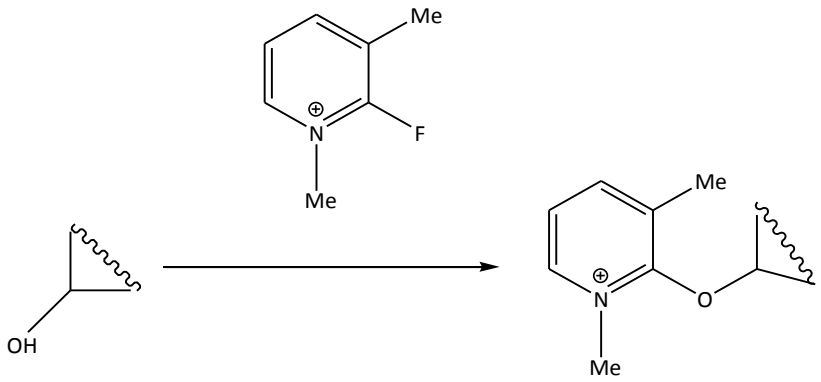


Figure 4.5 Example of the general reaction of derivatisation reagent with a phenolic OH group of the analyte (estrogen)

Selection of of a derivatisation reagent mainly depends on the functional group of the target analyte and corresponding mechanism of the derivatisation reactions; there is

variety of derivatization reagents available for reaction with the following functional groups in the target compounds.

Ketones and aldehydes

The ionization capability of ketone and aldehyde functional group containing compounds is very low in electrospray ionization therefore a chargeable group has to be introduced into the structure to increase the ionization. The most widely used reagent is HMP (2- Hydrazino-1- methyl pyridine). Other examples include Girard's reagent, hydroxyl amine and DNPH (2, 4-Dinitrophenylhydrazine) [113, 119].

Alcohols and phenols

Alcohols and phenols are considered as neutral compounds and their ionisation efficiency is low so in order to increase the response in the MS, derivatisation is commonly employed to enhance the ionization of the target compounds. The common example used of a reagent in this group is dansyl chloride. Other examples include picolinic acid and isonicotinyl azide [113, 119]. .

Carboxylic acids

Carboxylic acids can be detected in the mass system in negative ion mode with very low detection sensitivity. The compatibility of the mobile phase with ESI-MS for carboxylic separation is not very good, so derivatization of carboxylic acid before analysis is an option where the carboxylic acid can be derivatised so that it carries a

positive charge. Examples of such reagents include Trimethylaminoethyl and 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole) have been recently introduced for use in the derivatization of carboxylic acids [113, 119].

Amines

There are many problems associated with the analysis of amines on LC-MS including polarity, high water solubility and basicity along with the high matrix effect and background noise. Derivatisation of amines produces more hydrophobic compounds which can be easily separated and detected by LC-MS. Example of derivatisation reagents include 4-fluoro-7-nitro-2, 1, 3-benzoxadiazole, acetic anhydride, naphtyl isothiocyanate and 4-(trimethylammonium) anilyl-*N*-hydroxysuccidimidyl carbamate iodide [113, 119].

Thiols

Thiols are generally very unstable compounds and undergo oxidation during the sampling procedures. The stability of thiols can be increased by derivatization procedures by the introduction of a charged moiety lead to increased detection sensitivity in the mass system. The reagents used for derivatisation of thiols include ω-bromoacetonylquinolinium bromide, 2-bromo-4'-chloroacetophenone, 2-bromo-4′ -bromoacetophenone, Iodoacetamide, isopropylchloroformate and Ellman's reagent [113, 119].

4.1.8 The importance of the determination of estrogens in the body

There is significant evidence that endogenous estrogens have a vital role in the development of breast cancer. The risk of the cancer is more in women having either high levels of blood and urinary estrogens or exposed to increased estrogens [120]. Thus there has been an increased interest recently in determination of endogenous estrogens because of their links to breast, ovarian and prostate cancer with more interest in ovarian cancer because epidemiological studies suggest the role of postmenopausal estrogens in these diseases [121, 122].

Determination of estrogens especially estradiol is essential in assessing female reproductive functions including menopausal status, oligoamenorrhea and infertility status. The concentration is also monitored during ovulation induction and *in vitro* fertilization preparation. The analysis of estrogens is also very important in the determination of inborn errors in metabolism of sex steroids, puberty disorders, therapeutic drug monitoring in case of low dose female hormonal replacement therapy, male osteoporosis, cerebrovascular diseases, Alzheimer's disease and deficiency of estrogens in men [105, 123].

Measurement of estrogens also plays a vital role in the diagnosis of endocrine disorders. Accurate measurement of these hormones is essential for the diagnosis of many endocrine disorders including Cushing's syndrome, premature adrenarche, hirsutism and adrenal insufficiency [124].

Estrogens also have a very important role in neuroprotective processes and it has been shown in postmenopausal women that estrogen hormone therapy reduced the rate of stroke related mortality. It also has been proposed that estrogens are beneficial in both pre- and post ischemic treatment [106].

The determination of steroids is also very important in sports for doping control. According to world anti-doping agency it is prohibited to use anabolic steroids in sports therefore it becomes very important to accurately measure the concentration of these steroids in athletes and players of other sports who are using these steroids to increase their physical strength [117].

Apart from measuring endogenous estrogens it is also very important to pay attention to other sources as well. A serious concern has been raised over the recent past regarding the environmental pollution due to endocrine disruptors. It has been thought that some feminizing compounds have severe adverse effects on both humans and aquatic creatures. It is therefore important to accurately measure these estrogenic compounds in different samples from the environment [118], quite a number of these compounds are also phenols such as nonanoyl phenol.

It is also thought that female hormone such as estrogens have a role in pulmonary arterial hypertension (PAH), however this role in PAH is still unclear [125].

4.1.9 Methods of measurement

The most important task and challenge in determination of serum estrogens is their low concentration in the body. They are found in the picograms/ml range in the body [104, 123, 126]. Many methods have been used for the analysis of steroids, each having its own advantages and disadvantages [105]. The methods used for the determination of endogenous estrogens include radioimmunoassay (RIA), enzyme immunoassay (EIA), high performance liquid chromatography combined with electrochemical detection and gas chromatography coupled with mass spectrometry [105, 120]. Both RIA and EIA were shown to be very sensitive but the accuracy, specificity and reproducibility of these methods was poor. Detection of estrogens and their metabolites by HPLC with electrochemical detection in hamsters and pregnant women where the level of estrogens increased up to 10 fold was found to be of low sensitivity and also the specificity and accuracy of determining estrogens and their metabolites in biological matrices by this method was not clear [120, 124]. On the other hand stable isotope dilution GCMS produced results that were accurate and specific however this method was time consuming and extremely laborious [120, 124] and in addition electron impact ionisation may produce many fragments thus reducing the sensitivity of the method.

Liquid chromatography coupled with mass spectrometry (electrospray ionization) is now extensively used in the analysis of steroids because of its accuracy, selectivity, versatility and capability for simultaneously quantifying multi analyte samples. The

ionization of a compound is essential in the MS and the ionization process takes place in the liquid phase in electrospray ionization. Liquid chromatography coupled with mass spectrometry has employed by a number of scientists for determination of steroids [104, 105, 106,115, 116, 117,121, 123, 124, 126, 127, 128, 129] and is considered to be superior to other methods and is the most powerful and reliable analytical tool to measure the concentration of endogenous estrogens in the body.

4.1.10 Aim of the study

This project was carried out in collaboration with the University of Glasgow. The aim was to determine the role of estrogens and their metabolites in pulmonary arterial hypertension. The detection of the estrogens and their metabolites in the body is a challenging task because of their low concentration in the body. So the main aim of this study was to develop a derivatisation reaction to determine the concentrations of estrogens and their metabolites and also to find a chromatographic method for separating isomeric metabolites of the estrogens. The sample to be analysed was the cell culture medium from pulmonary artery cell cultures following their incubation with estradiol.

4.2 Experimental

4.2.1 Chemicals and materials

2-fluoro-1, 3-dimethylpyridinium *p*-toluenesulfonate, a derivatizing reagent was purchased from Sigma-Aldrich, UK**.** Estradiol and estrone were also purchased from Sigma Aldrich, UK. The internal standards, labelled estrone $^{13}C_2$ and estradiol $^{2}H_5$ were obtained from Sigma Aldrich. Estrogen metabolites 1, 3, 5(10)-estratriene-3, 16α-diol-17-one (16alphaOHE1), 1, 3, 5(10)-estratrien-3, 16α, 17β-triol (16aplhaOHE2), 1, 3, 5(10)-estratrien-3, 16α, 17β-triol (16-epi-E3), 1, 3, 5(10)-estratrien-3, 17α-diol (17alphaE2), 1, 3, 5(10)-estratrien-3, 4-diol-17-one 4-methyl ether (4MeOHE1), 1, 3, 5(10)-estratrien-3, 4, 17β-triol 4-methyl ether (4MeOHE2), 1, 3, 5(10)-estratrien-2, 3-diol-17-one 2-methyl ether (2MeOHE1), 1, 3, 5(10)-estratrien-2, 3, 17β-triol 2-methyl ether (2MeOHE2), 1, 3, 5(10)-estratrien-3, 4, 17β-triol (4OHE2), 1, 3, 5(10)-estratrien-3, 4-diol-17-ONE (4OHE1) were provided by the University of Glasgow, UK. HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. AnalaR grade formic acid (98%) was obtained from BDH-Merck, UK. Methanol and triethylamine (TEA) were purchased from Sigma-Aldrich, UK. Strata C18-E (55 µm, 70 Å) cartridges were purchased from Phenomenex, Macclesfield, UK.

4.2.2 Preparation of derivatization reagent

A fresh solution of derivatization reagent, 2-fluoro-1, 3-dimethylpyridinium *p*-toluenesulfonate, 5mg/ml was prepared in acetonitrile prior to each experiment.

4.2.3 Preparation of different standards

Standard stock solutions of estradiol and estrone were prepared at 1 mg/ml in ACN. Different working solutions were then prepared by diluting the stock solution. Solutions of IS were also prepared at 1 mg/ml in ACN.

4.2.4 Preparation of samples and treatment

Cell culture media samples were prepared and provided by the University of Glasgow. The samples were then kept at -20^{o}C until analysis.

4.2.5 Extraction of estrogens from media samples

The samples were then extracted by using solid phase extraction. Different C18 columns were used in order to achieve the best results for extraction. The results obtained with the Strata X polymer cartridges were very good. The Strata X cartridges were pre-conditioned with 1 ml of methanol and 1 ml of water. The media samples were spiked with 10 ng of stable isotope labelled internal standards before extraction. Then the media samples (4 ml) were passed through the column followed by a washing step with water (1 ml). After washing with water, the cartridge was eluted with 2 ml of methanol. The eluant was collected and then evaporated to dryness under nitrogen. Calibration curves were prepared by making different concentrations of steroid mixture

and then derivatising with the reagent 2-fluoro-1, 3-Dimethylpyridinium p-toluenesulfonate.

4.2.6 Derivatization with 2-fluoro-1, 3-dimethylpyridinium p-toluenesulfonate

The derivatization reaction of estrone and estradiol with FDMPTS is shown in the figure 4.6 and figure 4.7. The derivatisation procedure is shown in figure 4.8 and was carried out by adapting the work of Mukaiyama et al [130] as follows:

To the dried sample was added 50 µl of 1% TEA in acetonitrile. Then 50 µl of freshly prepared derivatization reagent (5 mg/ ml in ACN) was added to it. The mixture was vortexed for 10 seconds and then incubated for 15 minutes at 40^{o}C. After incubation 50 µl of mobile phase component A (0.1% Formic acid) was added to the mixture and vortexed. A volume of 10 µl was then injected into LCMS for analysis.

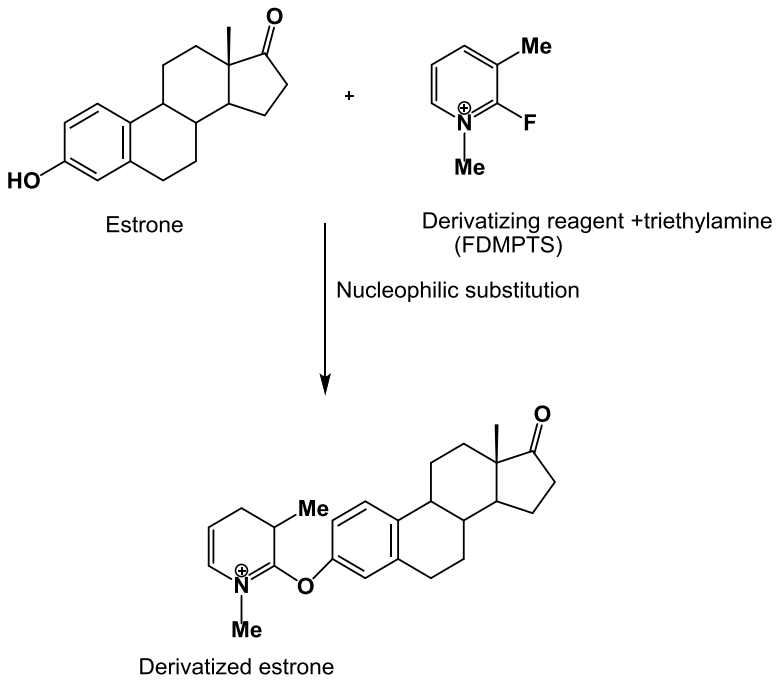


Figure 4.6 Derivatization of estrone with fluorodimethylpyridinium

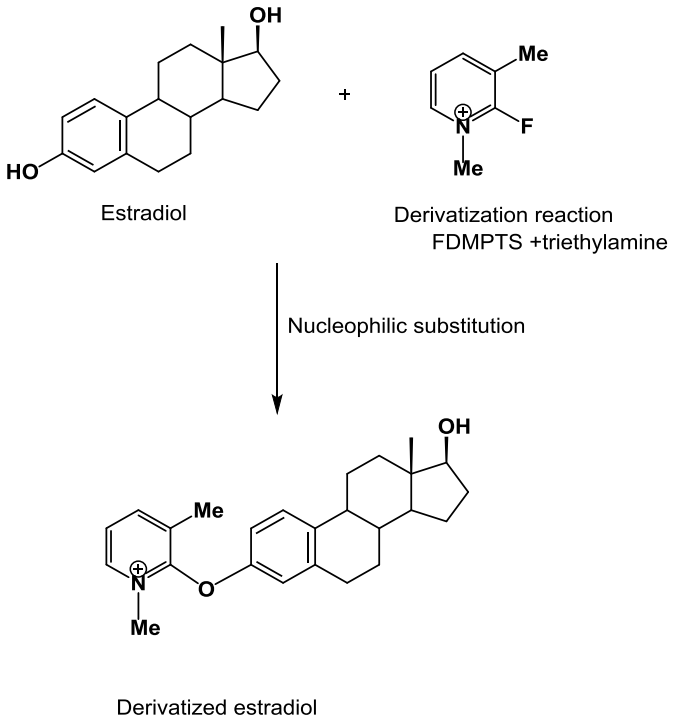


Figure 4.7 Derivatization of estradiol with the fluorodimethylpyridinium

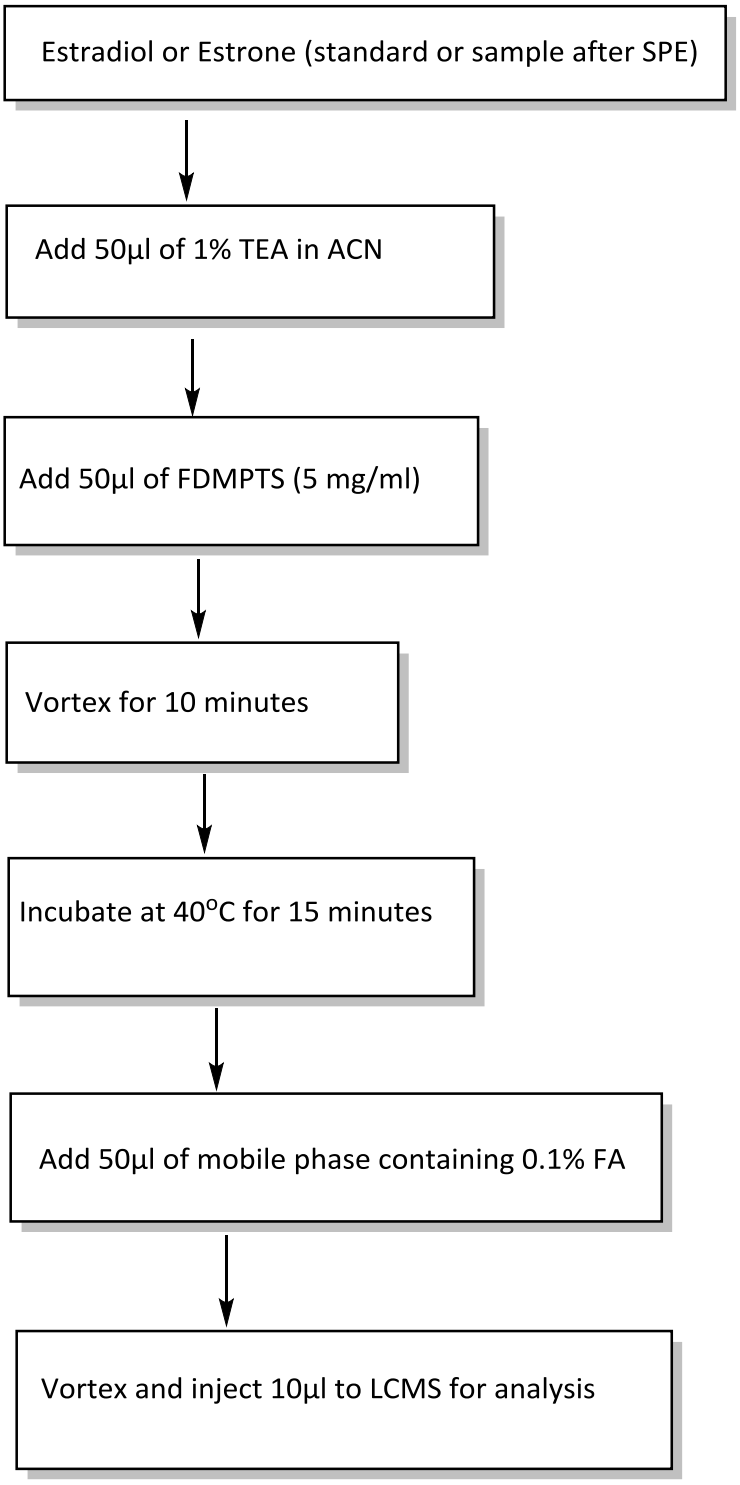


Figure 4.8 Flow chart for the derivatisation method

4.2.7 LC-MS analysis

Measurement of standards and samples was carried out on an LTQ Orbitrap mass spectrometer from Thermo Fisher Scientific (Hemel Hempstead, UK). The column used was an ACE 5 C18-AR column (150 × 4.6mm, 5µm Hichrom, Reading, UK). Mobile phase A consisted of 0.1% v/v formic acid in water and Mobile phase B consisted of 0.1% v/v formic acid in ACN which were used in a gradient method. The gradient was increased from 5% to 95% for Mobile phase B in 30 minutes and then decreased back to its initial composition in 1 minute. The system was re-equilibrated for 5 minutes before the next injection. The flow rate was 300 µl/min. The ESI interface was operated in a positive ion mode with a spray voltage of 4.5 kV. The temperature of the ion transfer capillary was 275°C and the flow rates of the sheath and auxiliary gases were 50 and 17 arbitrary units respectively. The full scan range was m/z 75 to 1200. The data was recorded using Xcalibur 2.1.0 software (Thermo Fisher Scientific). Mass calibration was analysed using the standard Thermo Calmix solution and the signal at 83.0604 m/z (2xACN+H) was selected as a lock masses for positive ion mode during each analytical run.

A Triple Quad LC/MS 6460 (Agilent technologies) was also used in the study. The column and mobile phase composition and gradient were the same as described above. The system was used in positive mode at a collision energy of 35 V using argon as a collision gas. The values for fragmentation and cell accelator voltage were set at 135 and 8 respectively. The column temperature was maintained at 30^{o}C. The data was recorded using mass hunter software Version B.06.00 (Agilent technologies).

4.3 Results and discussion

4.3.1 Optimisation of the derivatisation procedure

Preliminary runs of the underivatised steroids confirmed that the limits of detection were poor at *ca* 100 ng/ml. The reaction of steroids with FDMPTS produced a permanently charged derivative through nucleophilic substitution which has a high detection capability in the ESI mass spectrometer due to the inherent positive charge [126] which increases the detection sensitivity of the target analyte. This derivatization reaction is based on the Mukiyama reaction who for the first time described the phenomenon [130]. The reagent 2-fluoro-1, 3-Dimethylpyridinium *p*-toluenesulfonate was prepared in acetonitrile as described in the literature [118] both the analyte and reagent are completely dissolved in acetonitrile as compared to other organic solvents such as dichloromethane where the reaction fails to complete. The important parameters for the complete derivatization reaction were the quantity of derivatization reagent and triethylamine (base catalyst). Excess reagent and triethylamine were optimised at 5 mg/ml and 50 µl respectively and the excess was necessary to compensate for any impurities present in either samples or reagents such as traces of moisture. The derivatization reagent which is very hygroscopic needs to stay dry and therefore fresh solution was used every time for the analysis. The reagent upon exposure to air can trap moisture resulting in aggregation and stickiness of the reagent which ultimately results in reaction failure. Also some of the reagent is sold at 85% purity [131] which is responsible for failure of the reaction. In the current case, reagent

with a purity of ≥95% was used. Generally in alcohols the proton on the OH group is a poor leaving group therefore alcohols do not follow SN2 reactions very efficiently. Also most alcohols have insufficient acidic or basic properties to produce stable ions by modifying the *p*H of the solution. Therefore the best way to produce the ES active form of alcohols is to employ the reactions where the oxygen atom reacts as a nucleophile. Alcohols can be derivatized into N-Dimethylpyridyl ether salts by nucleophilic substitution of fluorine of the 2-fluoro-1, 3-Dimethylpyridinium *p*-toluenesulfonate with the oxygen of alcohols [132]. The positive ion mass spectra of the estrogens and their metabolites are shown below. There is an intense peak for estradiol and estrone obtained after derivatization at m/z 378 and m/z 376 respectively. The hydroxy methyl metabolites of estrone were detected at m/z 406 after derivatization. Hydroxy estrone metabolites are observed at m/z 392 after derivatization. Estradiol hydroxyl methyl metabolites were detected at m/z 408 in both the standard and samples. Estradiol hydroxyl metabolites were detected at m/z 394. The various metabolites of estradiol were also observed by this derivatization technique and the mass spectra of some of the derivatives are shown in figures 4.9-4.12. The 1, 3-Dimethylpyridium derivative of estradiol and estrone corresponds in mass to $(M +106)^{+}$ where M is the mass of the estrogen, which is 272 and 270 in case of estradiol and estrone respectively. The labelled internal standard estrone C13 and estradiol D5 were also detected in both standards and samples at m/z 379 and 383 respectively (figures 4.13 and 4.14).

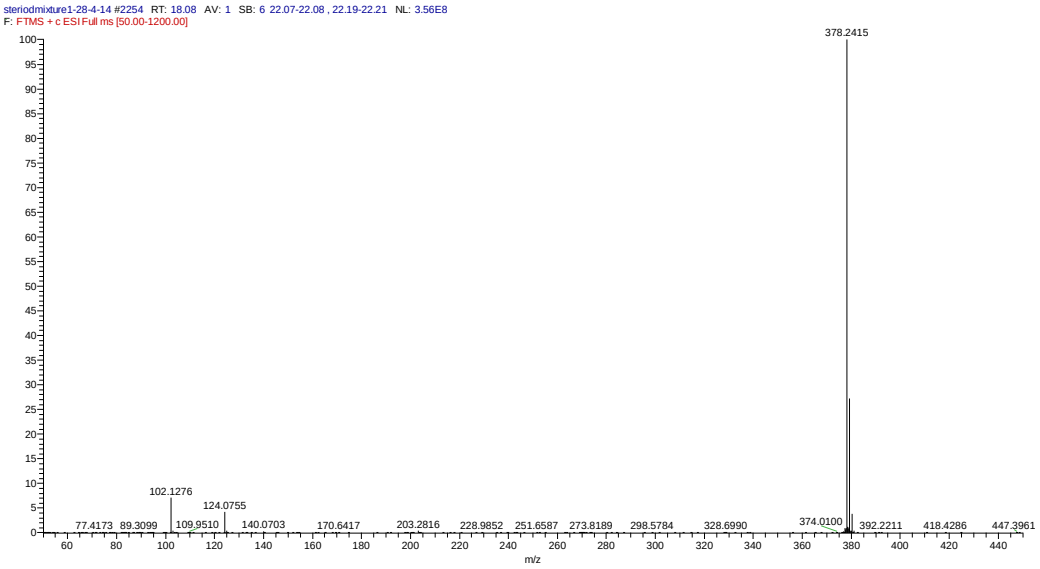


Figure 4.9 Mass spectrum of the DMP (fluorodimethylpyridinium) derivative of estradiol.

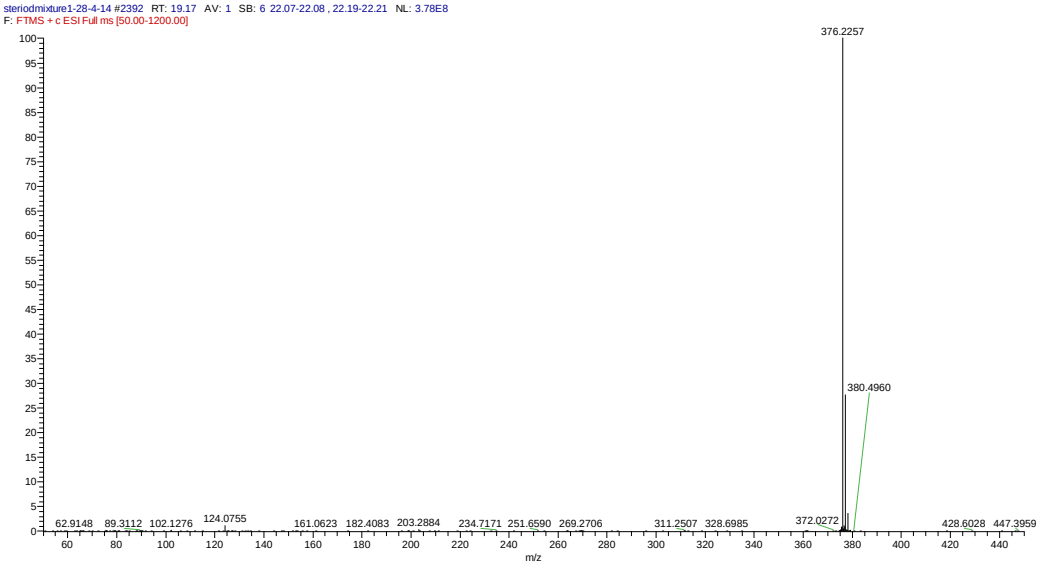


Figure 4.10 Mass spectrum of the DMP (fluorodimethylpyridinium) derivative of estrone.

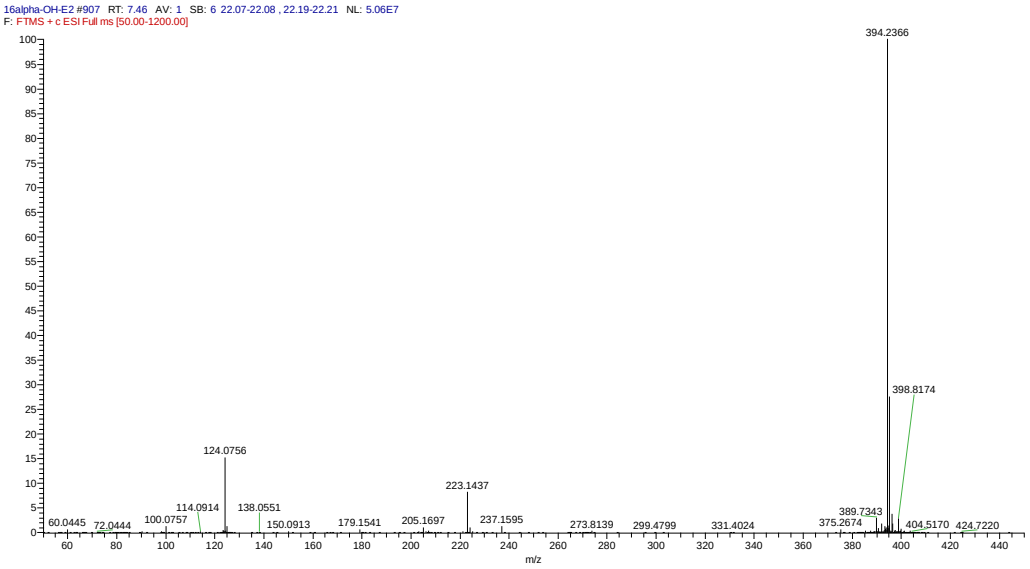


Figure 4.11 Mass spectrum of the DMP (fluorodimethylpyridinium) derivative of hydroxyestradiol.

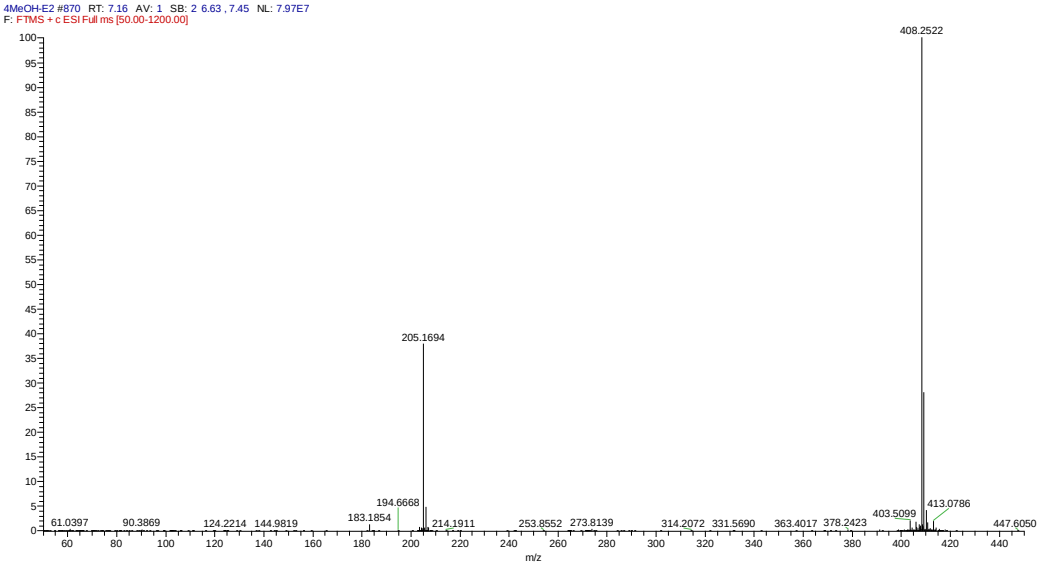


Figure 4.12 Mass spectrum of the DMP (fluorodimethylpyridinium) derivative of methoxy estradiol

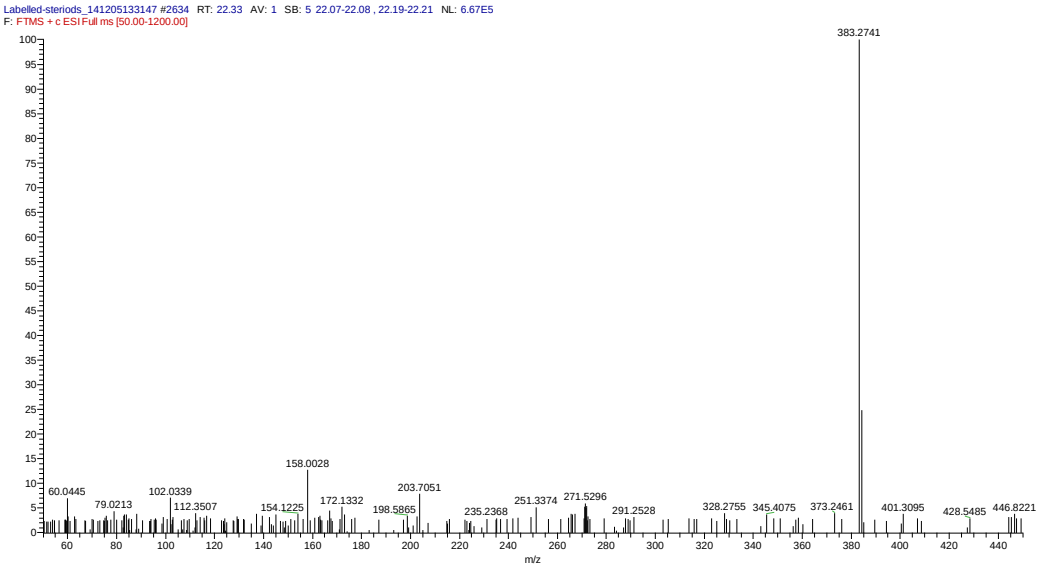


Figure 4.13 Mass spectrum of the DMP (fluorodimethylpyridinium) derivative of $^{2}H_{5}$- estradiol

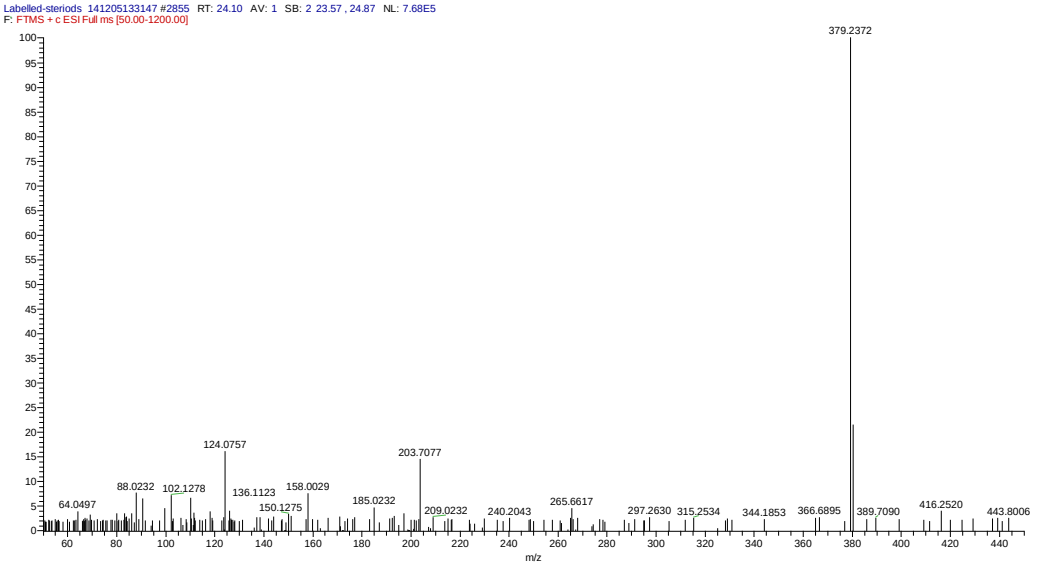


Figure 4.14 Mass spectrum of the DMP (fluorodimethylpyridinium) derivative of $^{13}C_{3}$- estrone

The method was optimised by selecting different strategies such as adding the triethylamine before the reagent which yielded improved results because it imparted the negative charge to the oxygen of reacting phenol so that it could react with the reagent immediately after it was added. Also initially 500µl of mobile phase was used in order to dilute the sample, because the concentrated sample could block the column but the results were not good with addition of 500µl mobile phase as some metabolites were not detected, so we used 50µl mobile phase to dilute the sample which improved the sensitivity of the method.

Several different types of columns were tested for their efficiency in separating the different estrogens. These included a ZIC-HILIC column, ACE Ultracore 2.5 Superphenylhexyl (150 × 3 mm id), ACE 5 PFP HILIC (PROTOTYPE) (250 × 4.6 id) and an ACE 5 C18-AR column (150 × 4.6mm, 5µm Hichrom, Reading, UK).

First we tried a Zic-Hilic column on orbitrap mass spectrometer, but the separation on the Zic-Hilic column was poor and there was no peaks observed for the estrogens on Zic-Hilic column (figure 4.15).

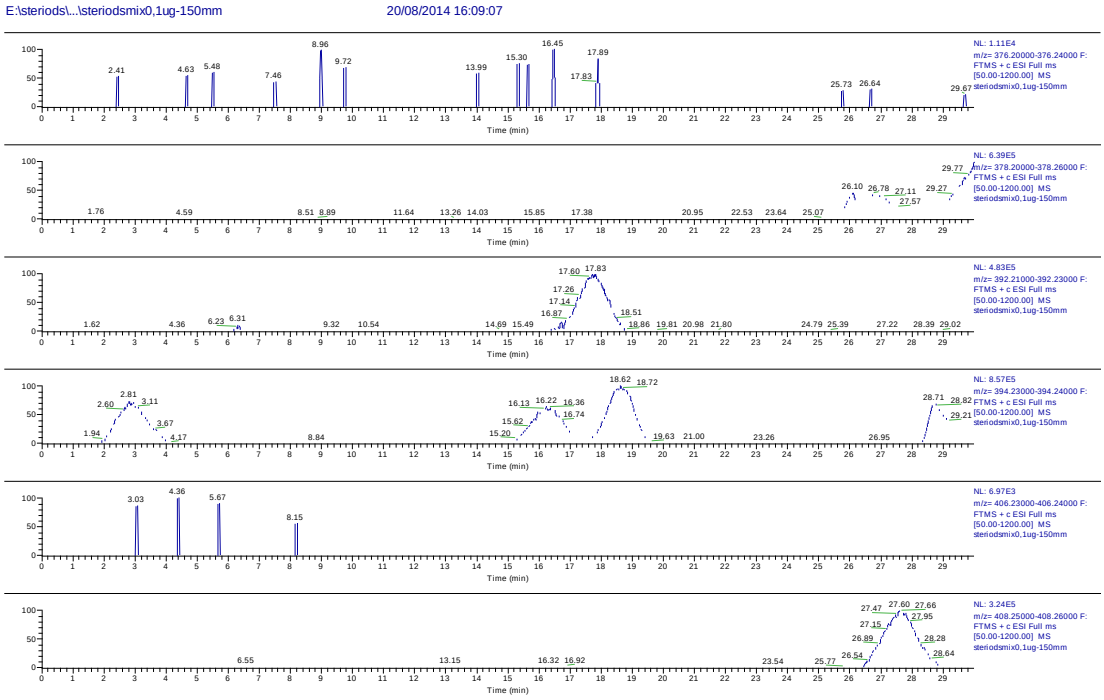


Figure 4.15 Chromatograms obtained from a ZIC-HILIC column for the derivatised estrogens. The separation on the ZIC-HILIC column was poor and there was no peaks observed for the estrogens on ZIC-HILIC column as seen in the figure.

Then after trying ZIC-HILIC we used ultracore phenylhexyl column, C-18 PFP and a HILIC (PROTOTYPE) (250 × 4.6 id) for the determination of estrogen and their metabolites. Although there was some separation of estrone and estradiol on phenlyhexyl column and C-18 PFP, the overall results were not consistent and satisfactory from these columns. The chromatograms obtained from the phenylhexyl column, C-18 PFP and the HILIC (PROTOTYPE) are shown below (figures 4.16-4.18).

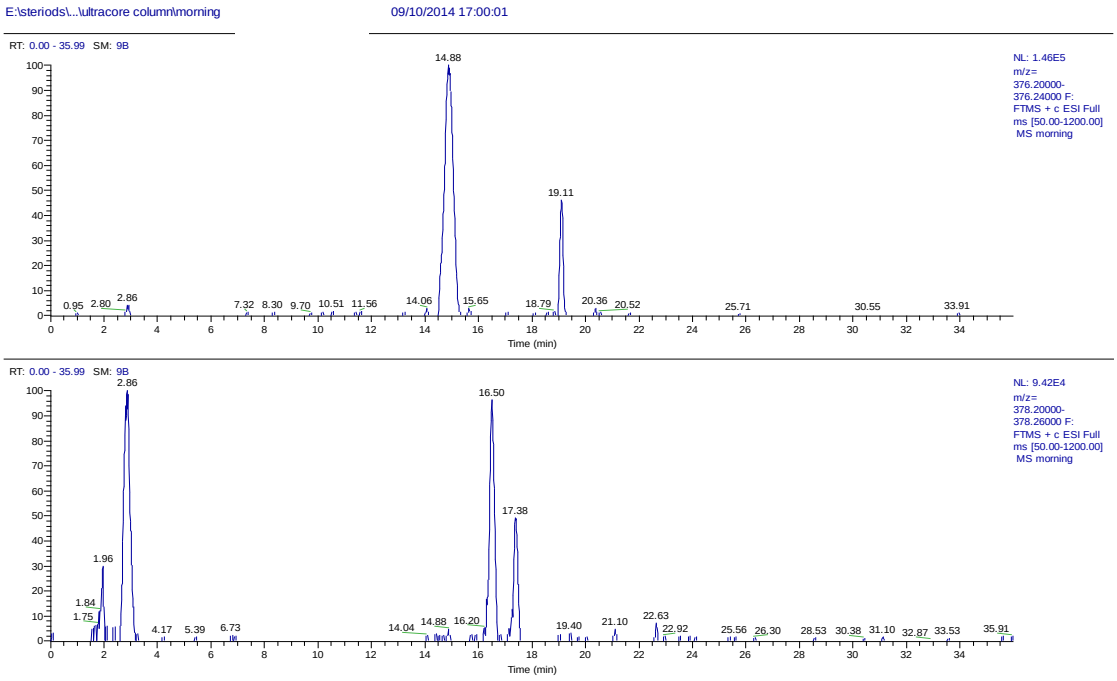


Figure 4.16 Chromatograms obtained from ultracore phenlyhexyl column. The results obtained from ultracore phenylhexyl column was not good and consistent, the separation was also poor and not matching the standard steroids mixture solution.

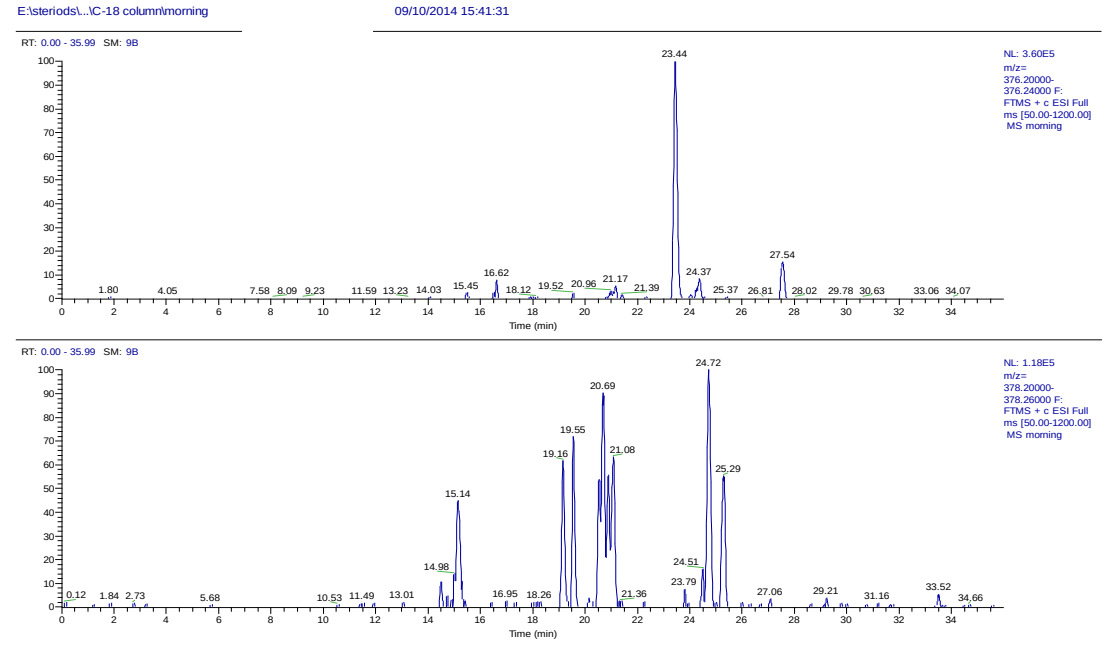


Figure 4.17 Chromatograms obtained from C-18 PFP. The results was not satisfactory, no peaks were observed for the estrogen and their metabolites

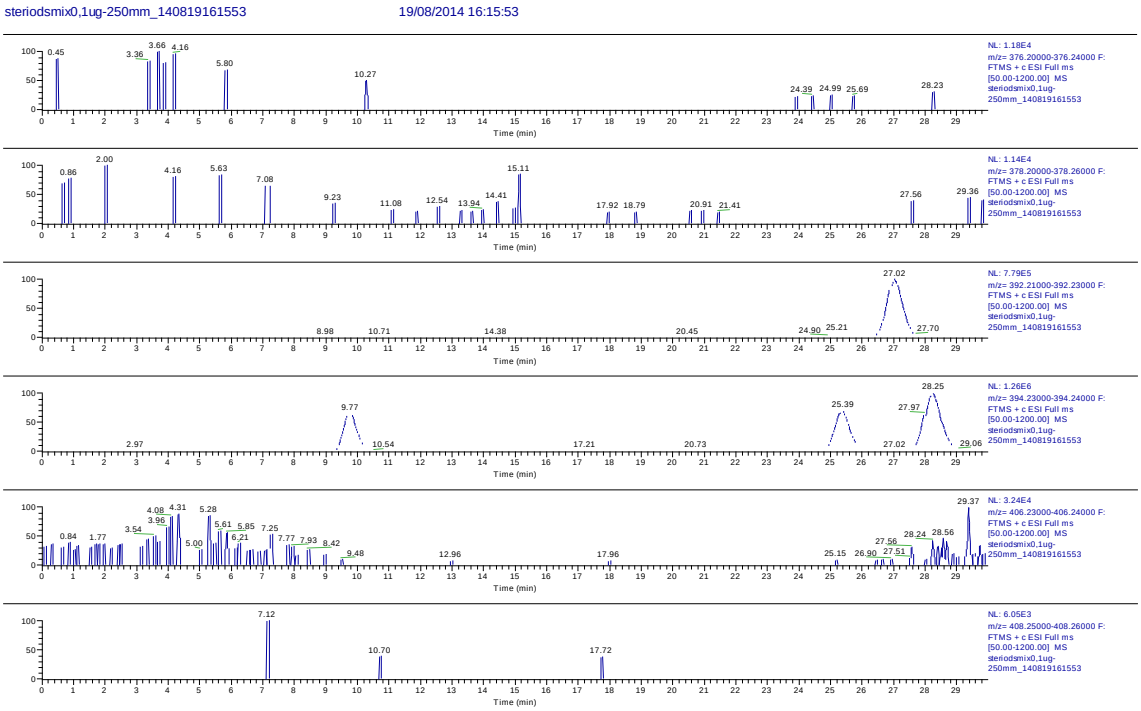


Figure 4.18 Chromatograms obtained from the HILIC (PROTOTYPE). The separation was very poor and no any peaks even observed with HILIC (PROTOTYPE) column.

Eventually satisfactory separation of the steroid mixture was obtained on a C18 AR column as shown in figure 4.19.

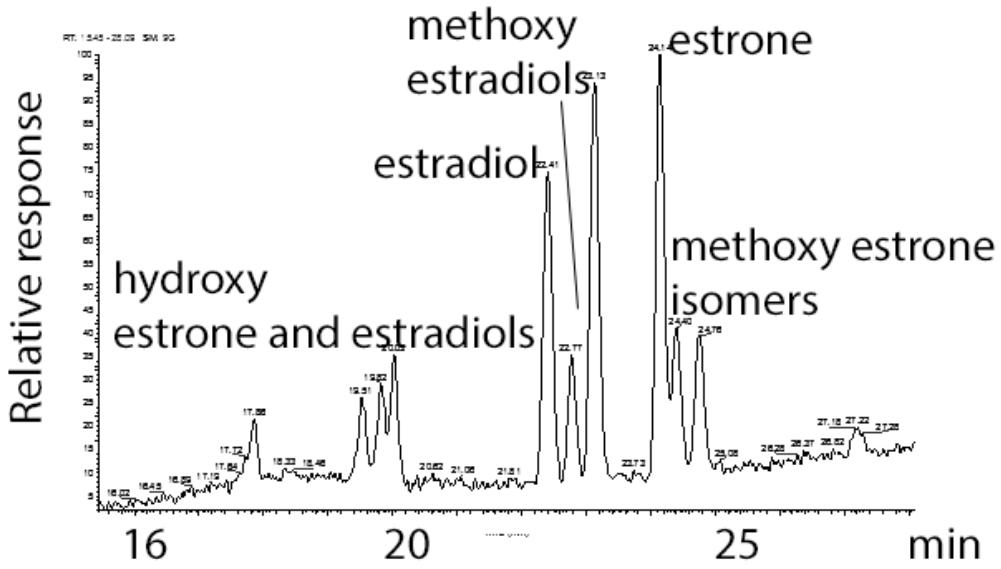


Figure 4.19 Separation of steroid mixture derivatised with DMP on a C18 AR column (conditions as in section 4.2.7), the separation obtained with C18 AR column was very satisfactory, both estrogens and their metabolites were observed and match the standard solution of steroids mixture and confirmed by x-calibur software.

Table 4.1 Summary of the columns used in determination of steroids

Column used	Conditions	Comments
ZIC-HILIC	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min Different composition of mobile phase A and B used isocratically	The separation on the ZIC-HILIC column was poor and there was no peaks observed for the estrogens on ZIC-HILIC column
Ultracore phenylhexyl column	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min	The results obtained from ultracore phenylhexyl column was not good and consistent, the separation was also poor.
C-18 PFP	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min	The results was not satisfactory, no peaks were observed for the estrogen and their metabolites
HILIC (PROTOTYPE)	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min	No any peaks even observed with HILIC (PROTOTYPE) column
C18 AR column	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min	The results obtained was very good and satisfactory, both the samples and standard have the same retention times, the estrogen and their metabolites was detected in both media samples and standards solution and the results is also reproducible. So C 18 AR column was selected for this study

Initially a quantitative method was developed by using standards spiked into water in order to determine limits of detection for the steroids. First of all estradiol and estrone were used to test the method. A calibration curve series was constructed over the range 1 ng/ml -16 ng/ml. The calibration curve and chromatograms of estrone and estradiol within this range are shown in figures 4.20-4.22.

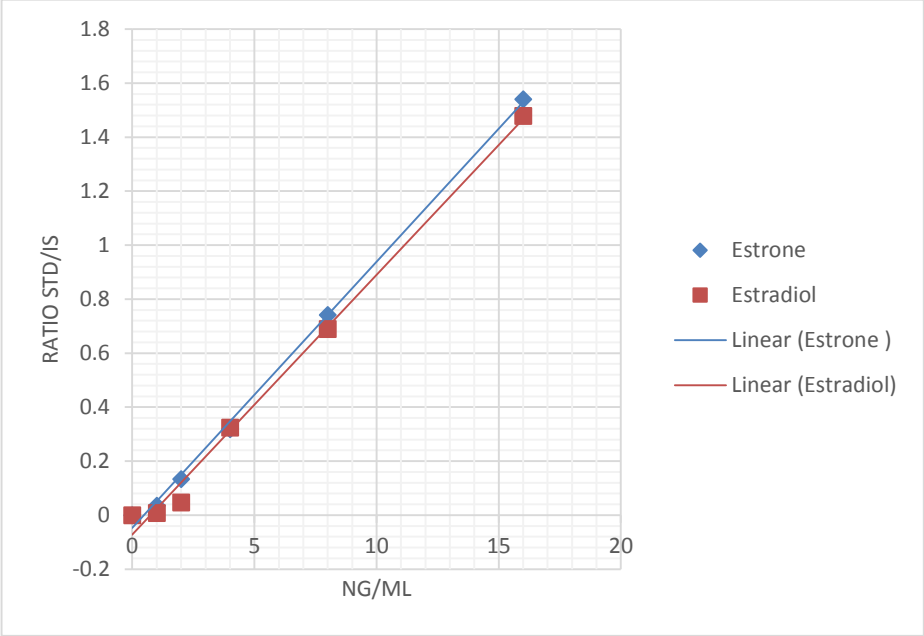


Figure 4.20 Calibration curve for estrone and estradiol in the range 1 -16 ng/ml

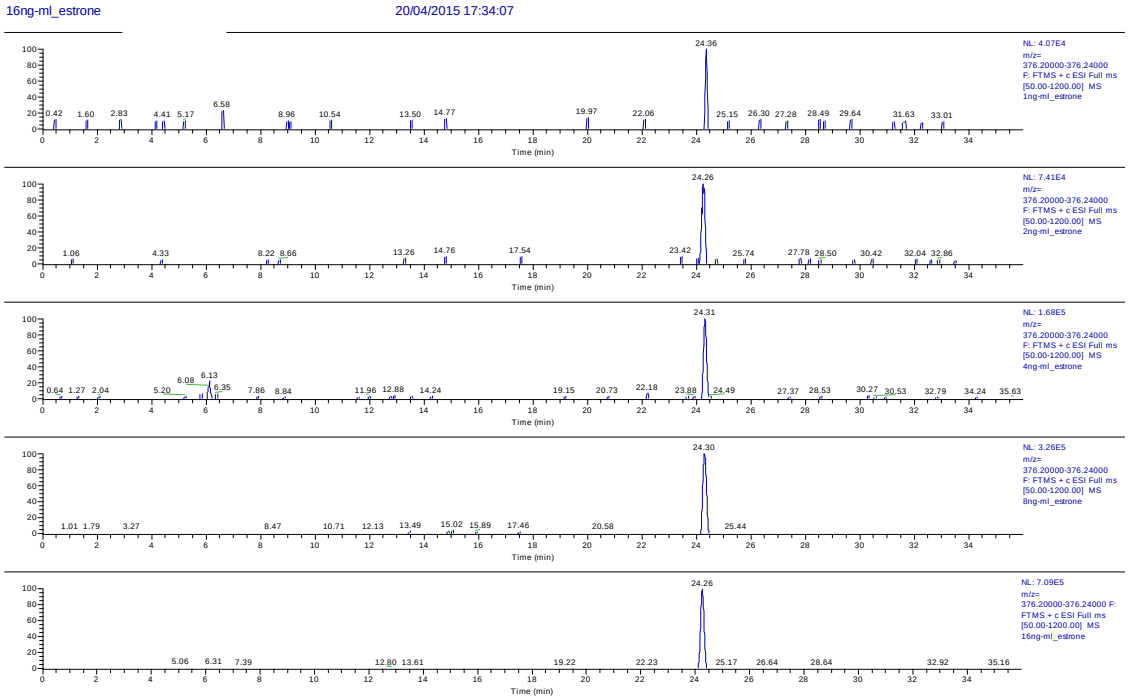


Figure 4.21 Extracted ion chromatograms for estrone in the concentration range of 1-16 ng

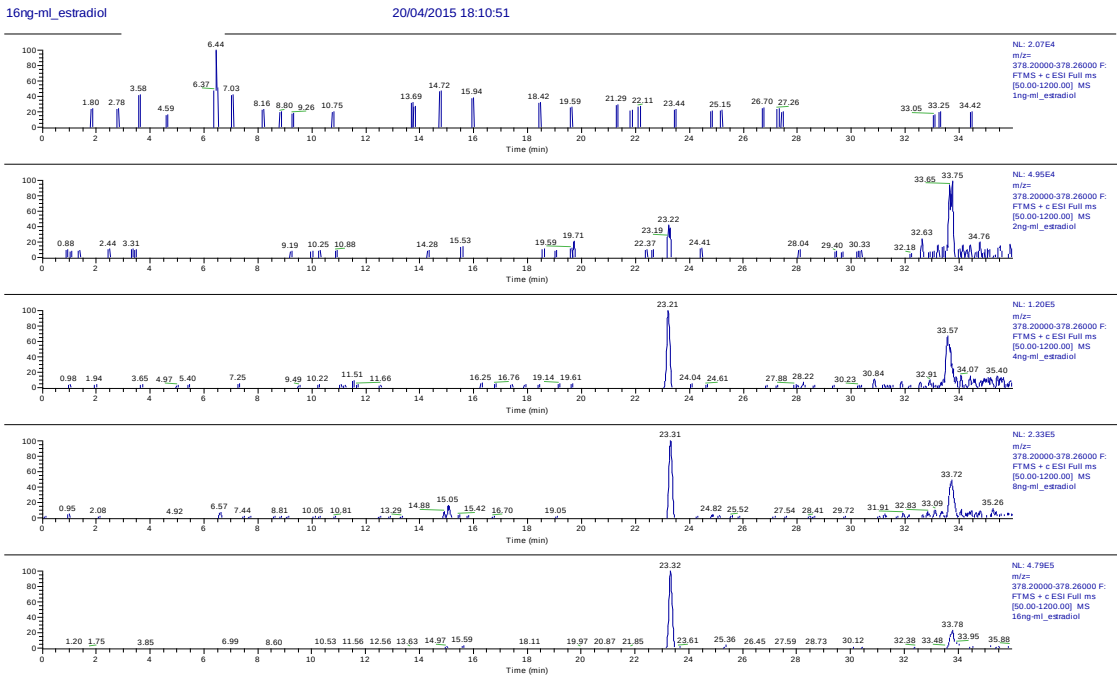


Figure 4.22 Extracted ion chromatograms of estradiol in the concentration range of 1-16 ng.

Then different concentrations of estrone and estradiol and their metabolites ranging from 0.1 ng/ml to 6.4 ng/ml were prepared in a mixture and derivatized with the reagent according to the method mentioned above, and were analysed by LC-MS. Calibration curves and chromatograms of estrone, estradiol and their metabolites in a steroid mixture are shown below (figures 4.23 to 4.30). The LOD was around 0.2 ng/ml for all the steroids apart from estrone and estradiol which could be detected at 0.1 ng/ml.

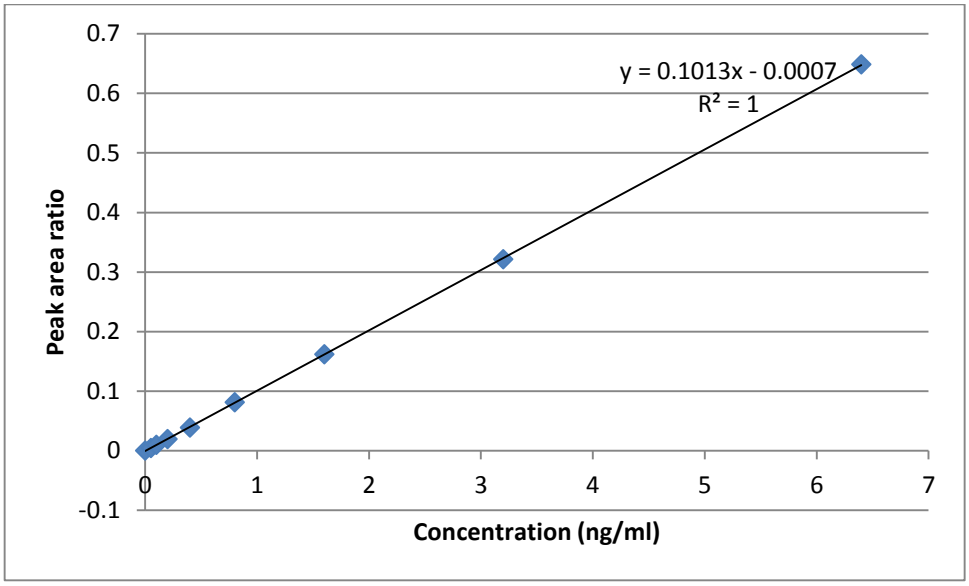


Figure 4.23 Calibration curve of estrone in steroid mixture

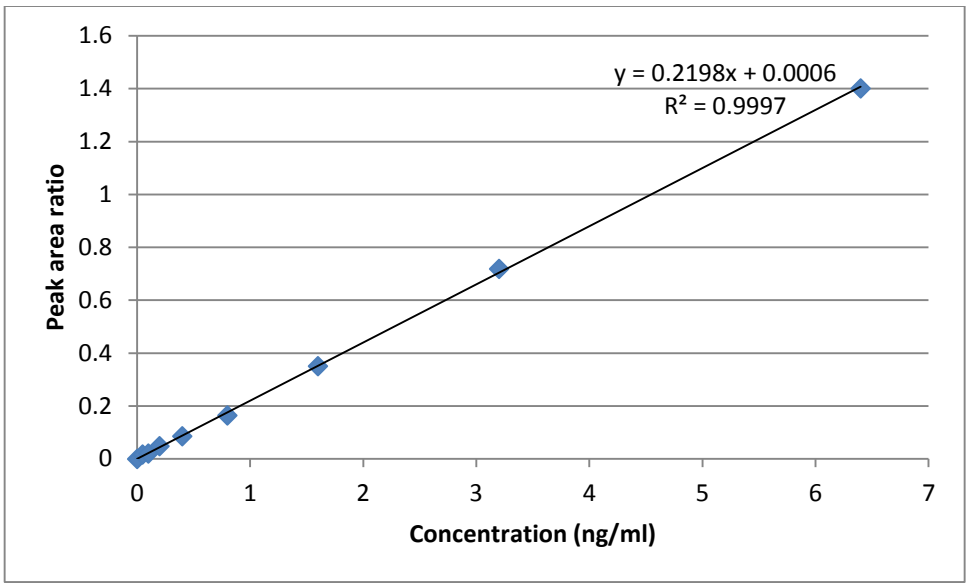


Figure 4.24 Calibration curve of estradiol in the steroid mixture

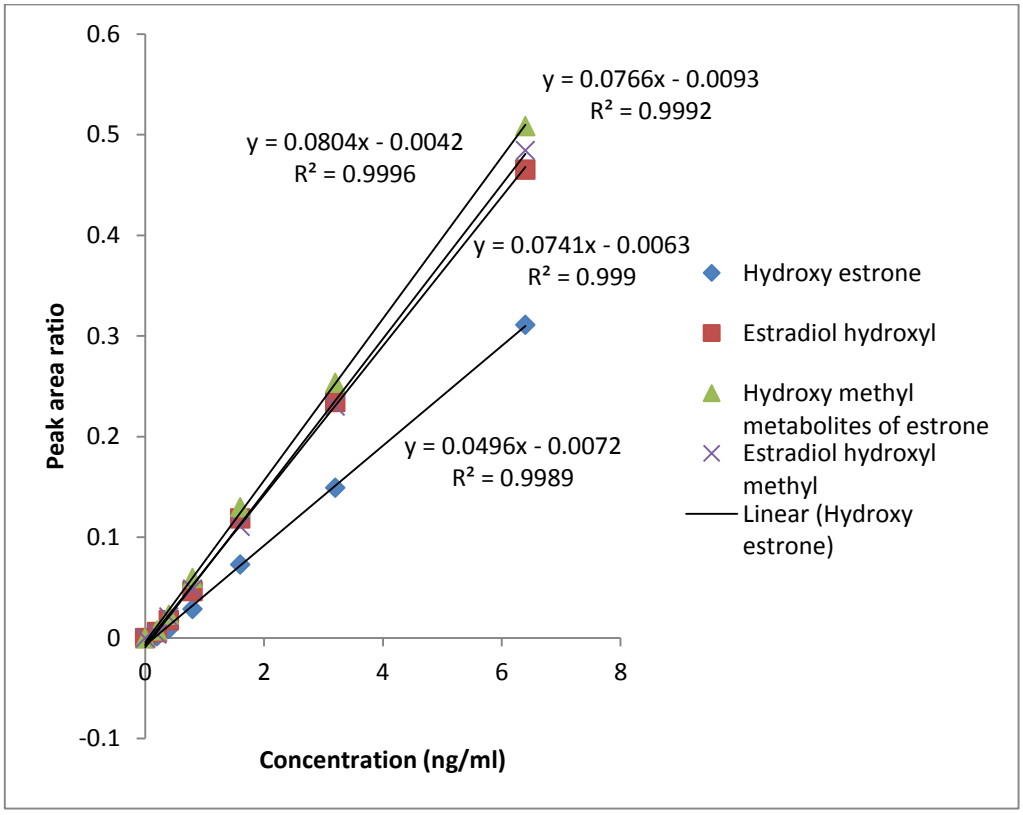


Figure 4.25 Calibration curves of different metabolites of estradiol in the steroid mixture

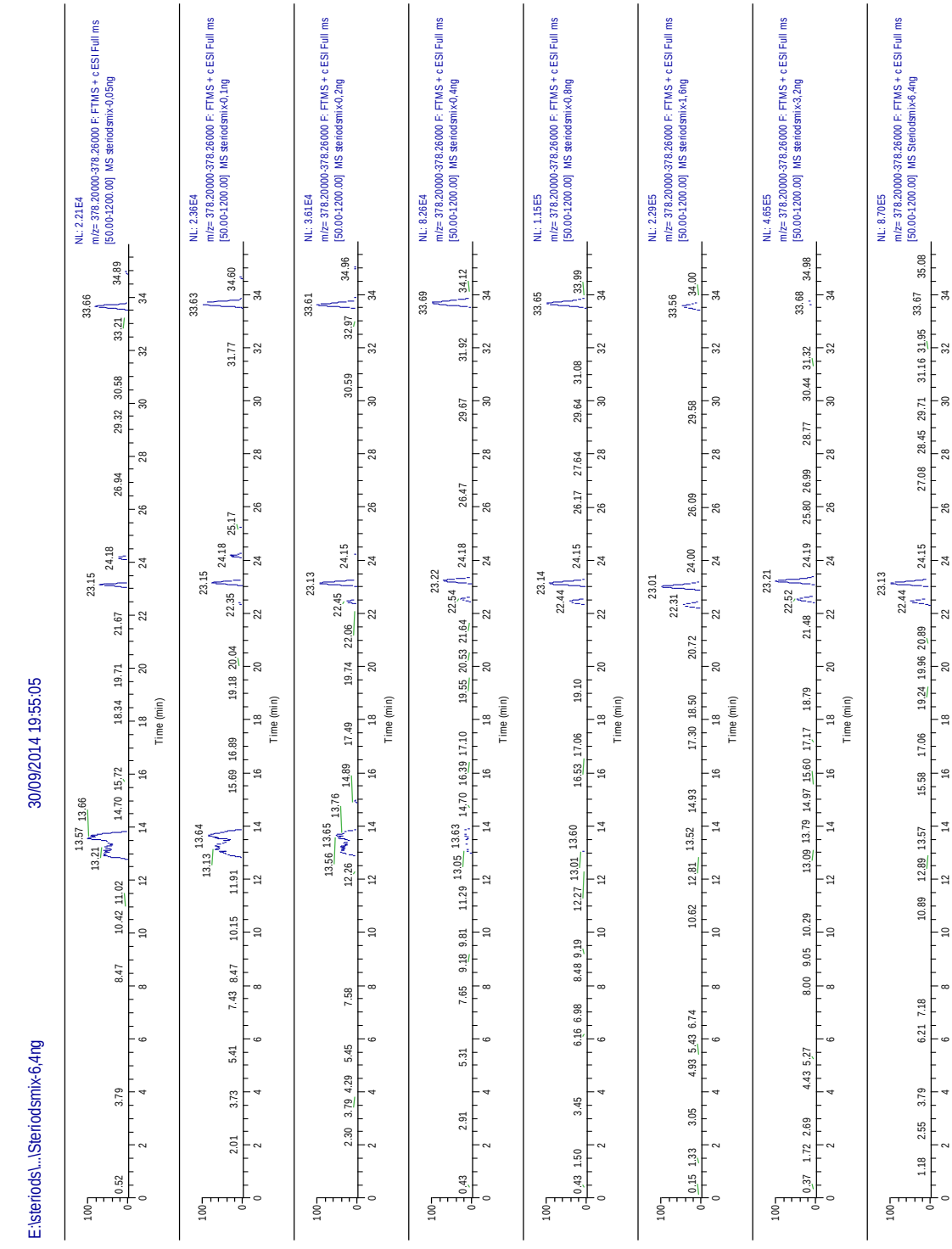


Figure 4.26 Chromatograms for derivatised estradiol at the different concentrations used to prepare the calibration curve.

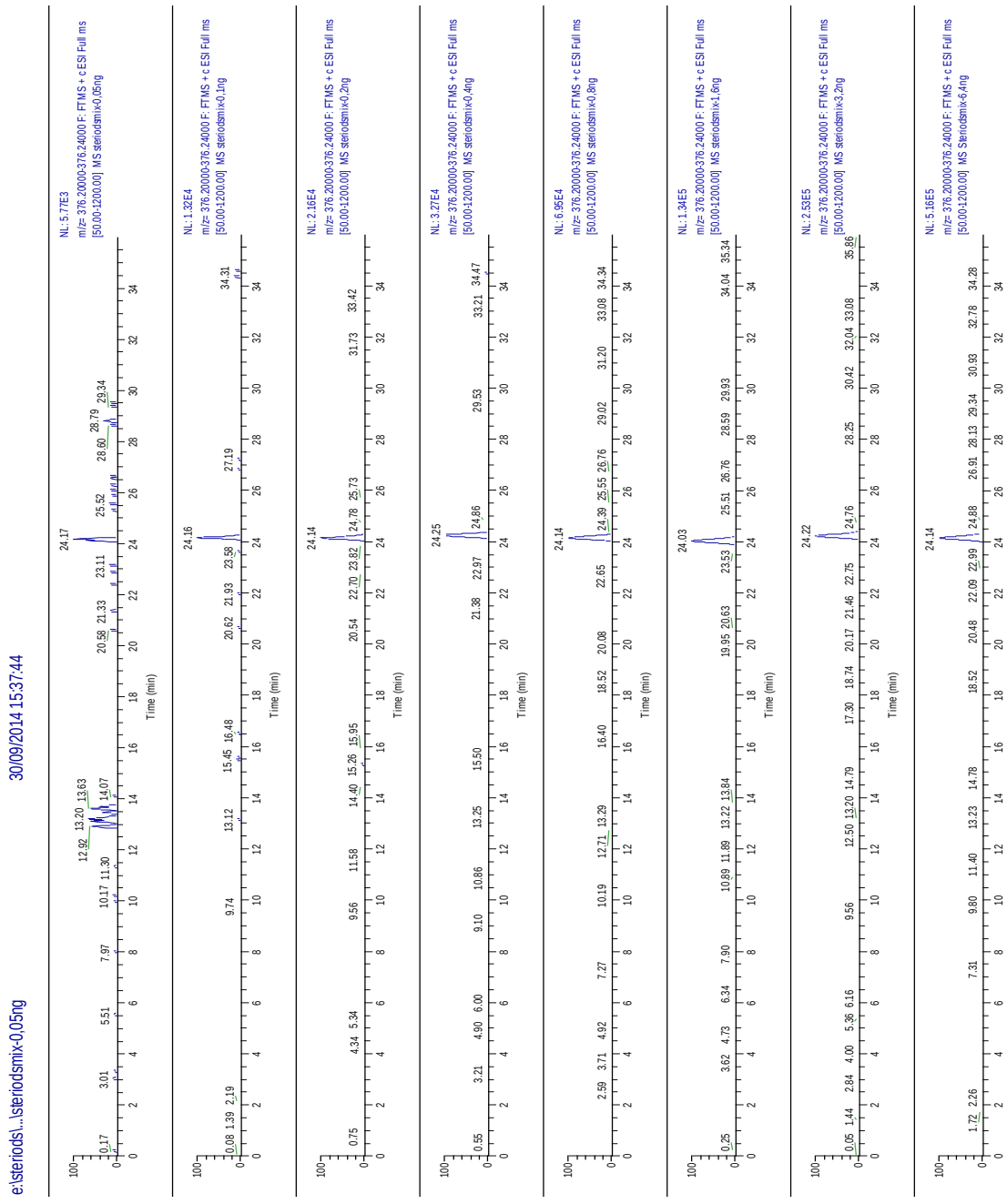


Figure 4.27 Chromatograms for derivatised estrone at different concentrations used to prepare the calibration curve.

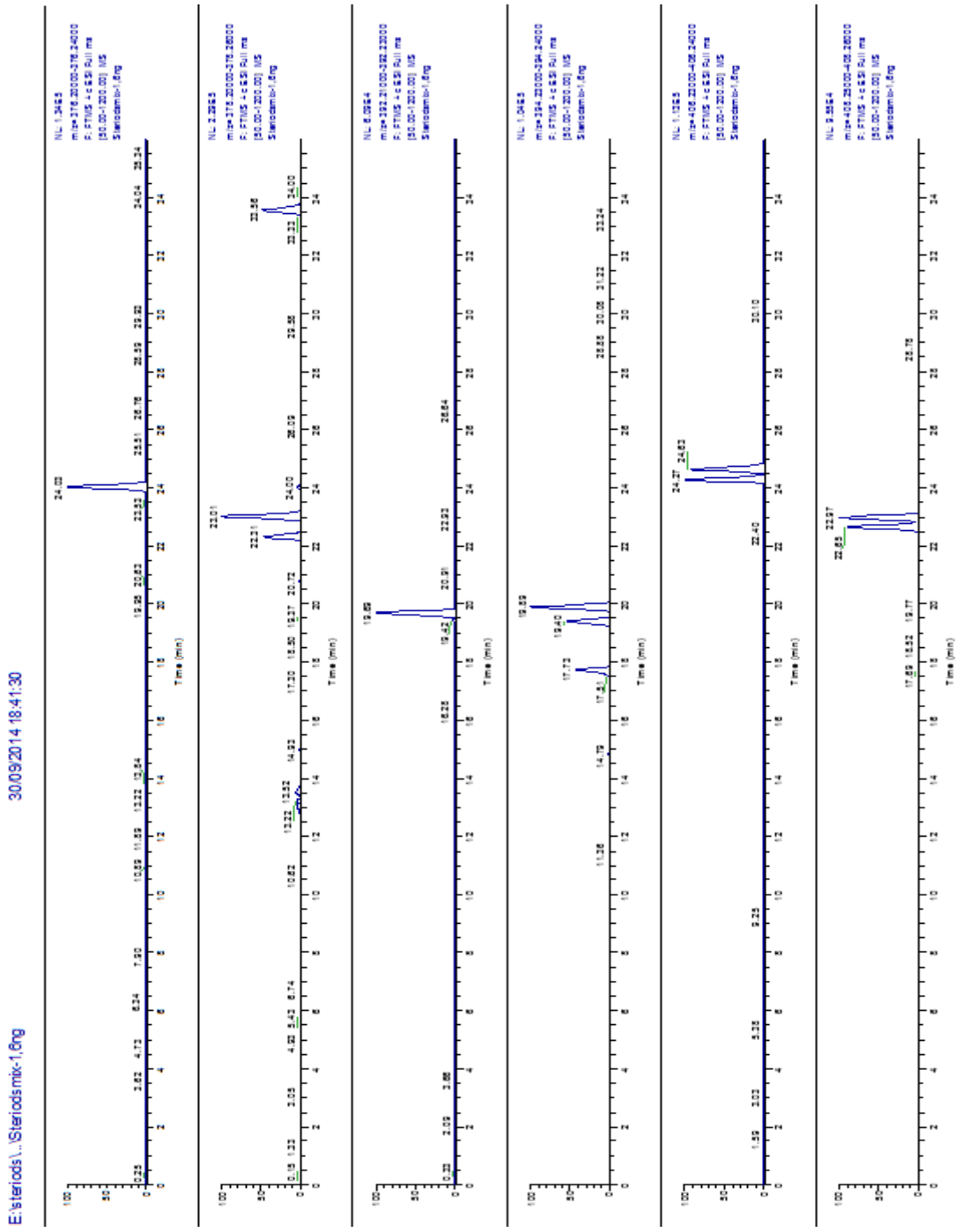


Figure 4.28 Extracted ion chromatograms of different metabolites of estrogens at the highest concentration of calibration curve 6.4 ng/ml. From the top estrone, estradiols, hydroxyl estrone, hydroxyestradiols, methoxy estrone, methoxy estradiol.

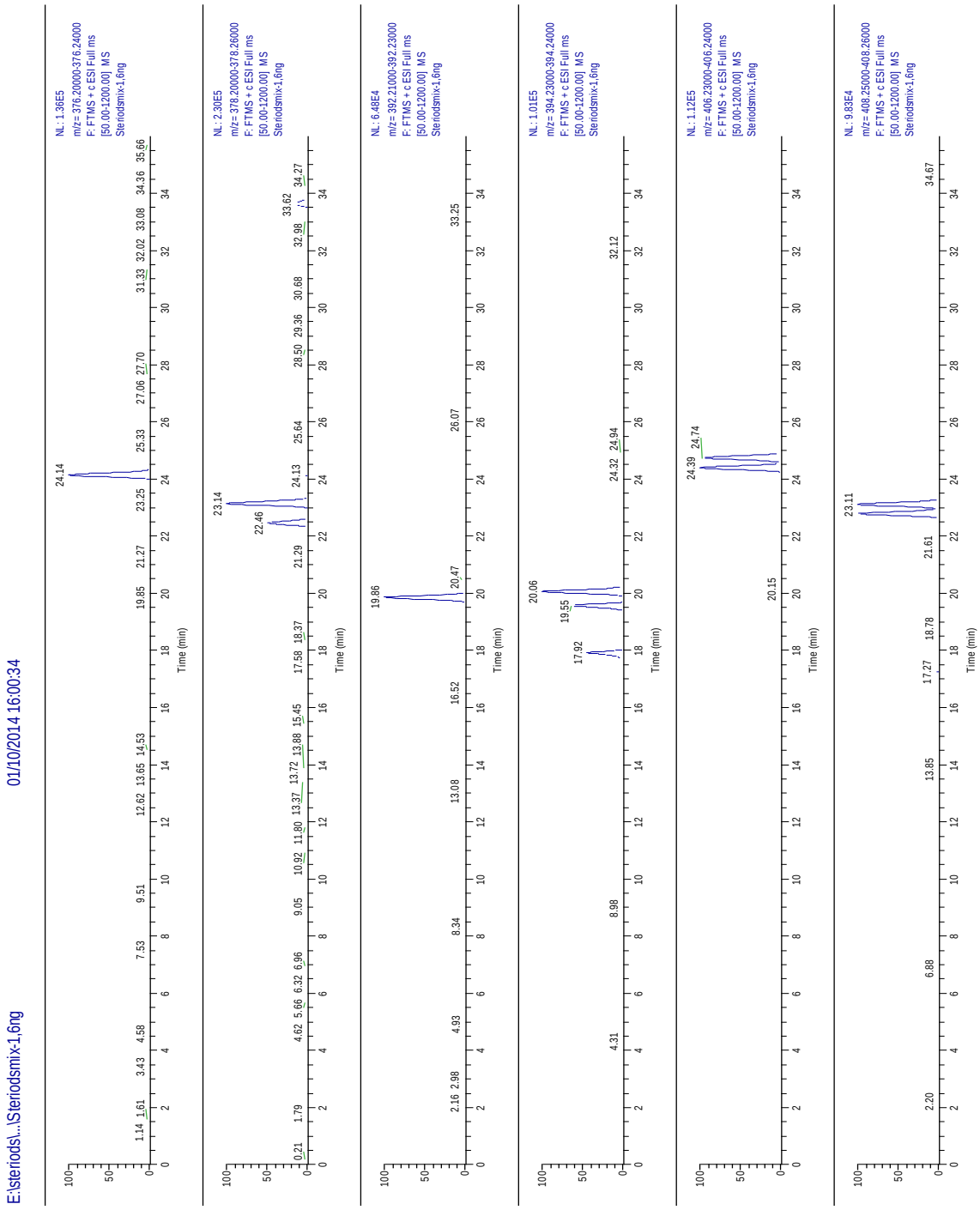


Figure 4.29 Extracted ion chromatograms of estrone, estradiol and their metabolites at a concentration of 1.6 ng/ml.

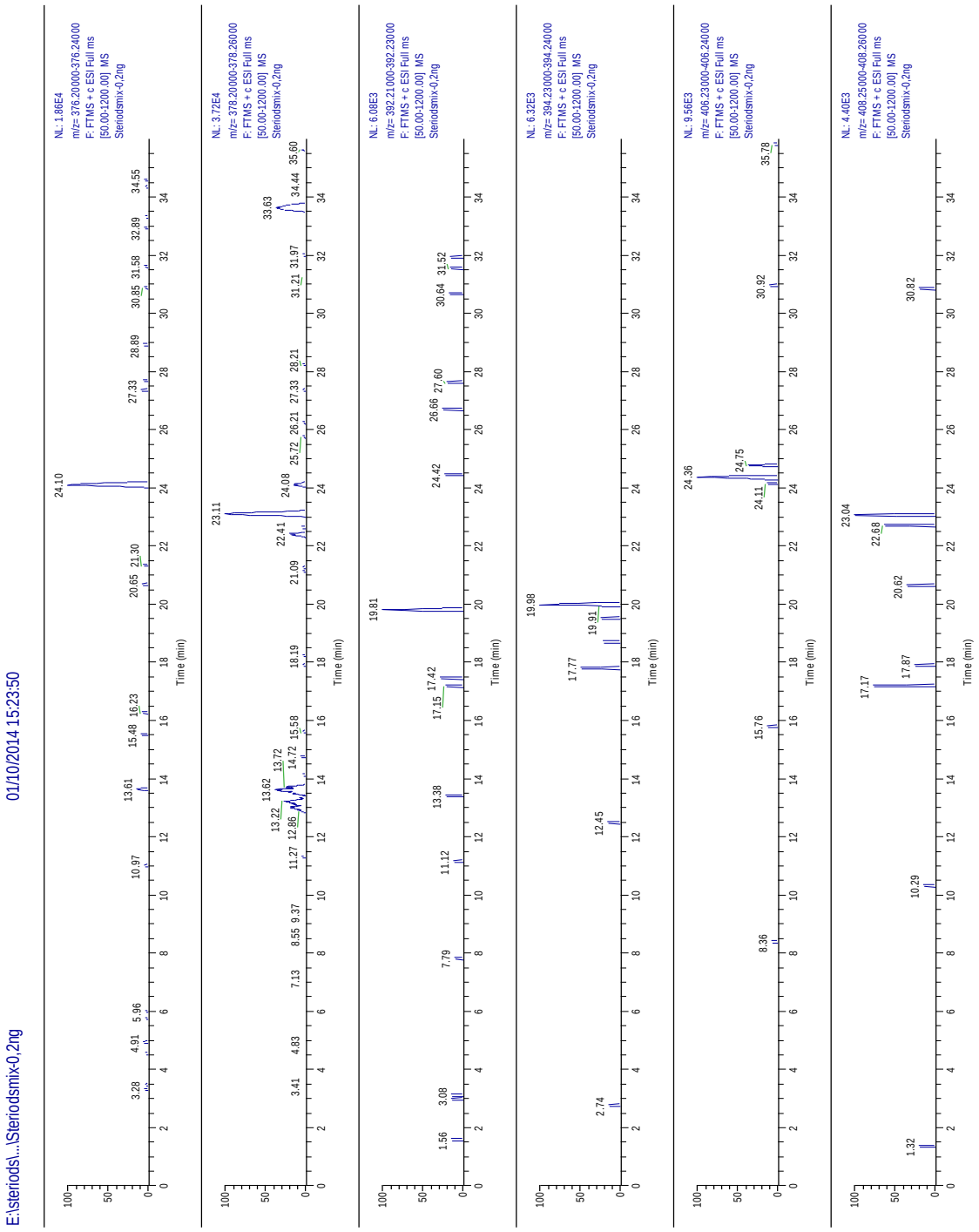


Figure 4.30 Chromatograms of different metabolites of estrogens at the LOD of 0.2 ng/ml.

Precision of the method was determined by selecting three concentrations and running them in triplicate. The chromatograms and detection sensitivity of higher and intermediate concentrations was good as compared to the lower concentration. The RSD determined for estrone at higher (1.6ng), intermediate (0.2ng) and low level (0.05ng) was ±1.9, 14.4 and 69.1 percent respectively, while the RSD determined for estradiol was ± 1.3, 1.7 and 64.0 percent respectively at three different concentrations. Thus the method was almost sensitive enough for requirements. The plan was to develop the method as far as possible and then transfer it to the University of Edinburgh so that it could be run on a QTRAP 5500 instrument which would give higher sensitivity than the Orbitrap. Samples of plasma and urine were extracted by using SPE on C18 strata X cartridges in order to try to concentrate the very low levels of steroid so that they could be detected by the method. However, the method was not successftul for the extraction and detection of estrogens in plasma and urine. The chromatograms are shown in figure 4.31 and figure 4.32 and there were no peaks observed corresponding to estrogens and their metabolites.

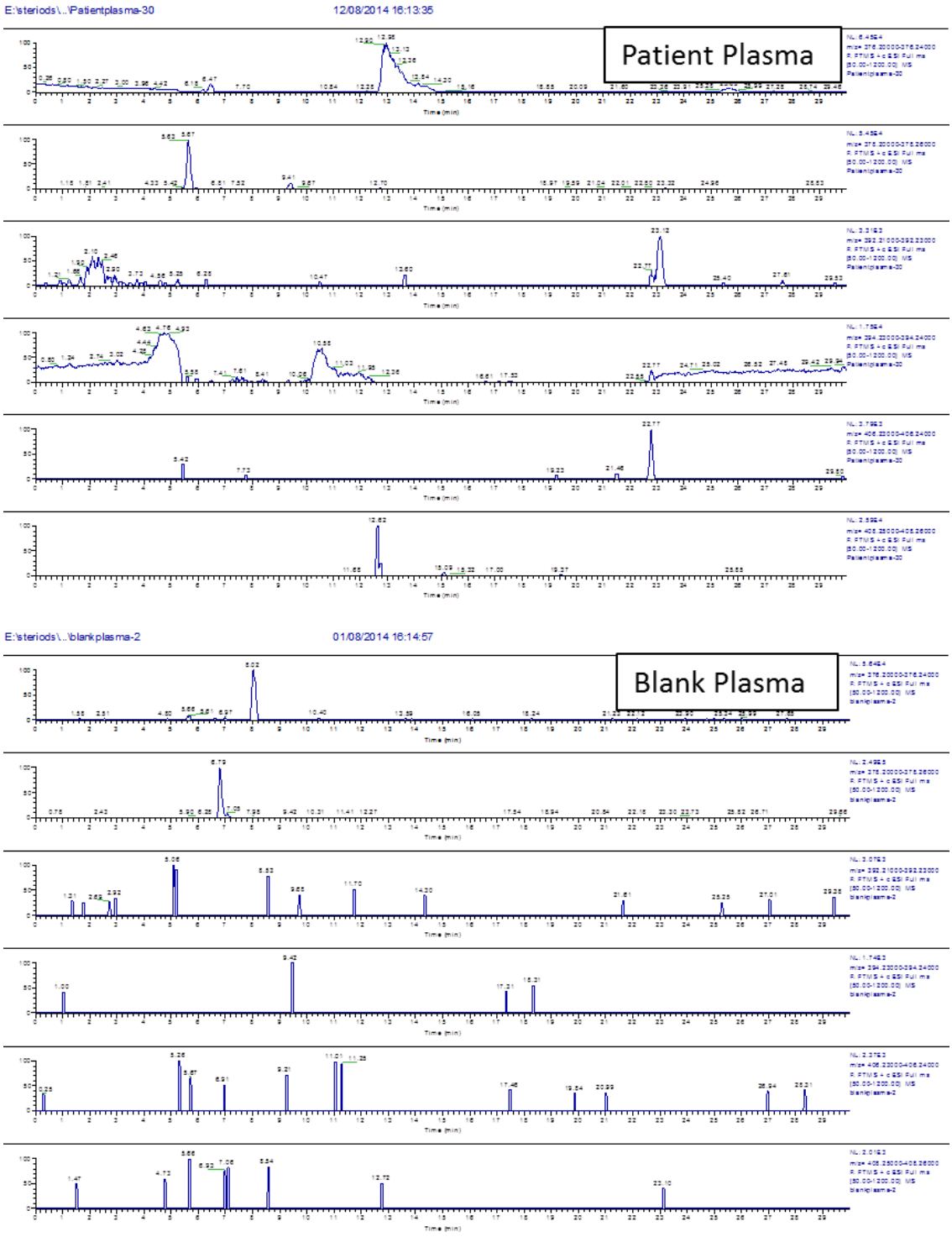


Figure 4.31 Chromatograms of estrone and estradiol and their metabolites obtained from extraction of plasma, the results were not satisfactory. There was no clear peaks observed for estrogen and their metabolites.

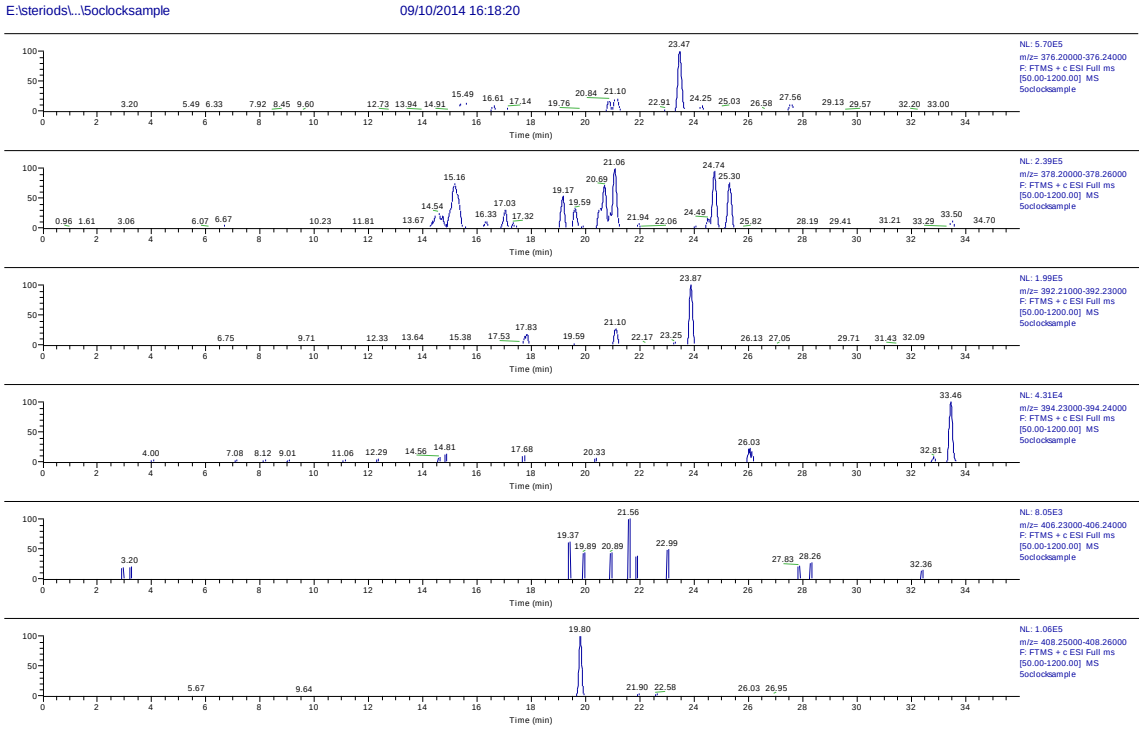


Figure 4.32 (cont) Chromatograms of estrone and estradiol and their metabolites obtained from extraction of plasma, the results were not satisfactory. There was no clear peaks observed for estrogen and their metabolites.

Samples were also run on an Agilent QQQ instrument but the LODs were only slightly better than on the Orbitrap. Figures 4.33-4.36 show the MS/MS spectra obtained with a collision energy of 35 V on the QQQ system for estrone, estradiol, methoxy estrone and methoxy estradiol. The major fragment ions in all cases were reagent specific at m/z 122 or m/z 124. Figure 4.37 shows a SRM trace monitoring the transition from m/z 376 to m/z 266 for estrone at a concentration of 0.1 ng/ml. However, it was unclear if this was genuinely 0.1 ng/ml since the sample blank contained similar levels.

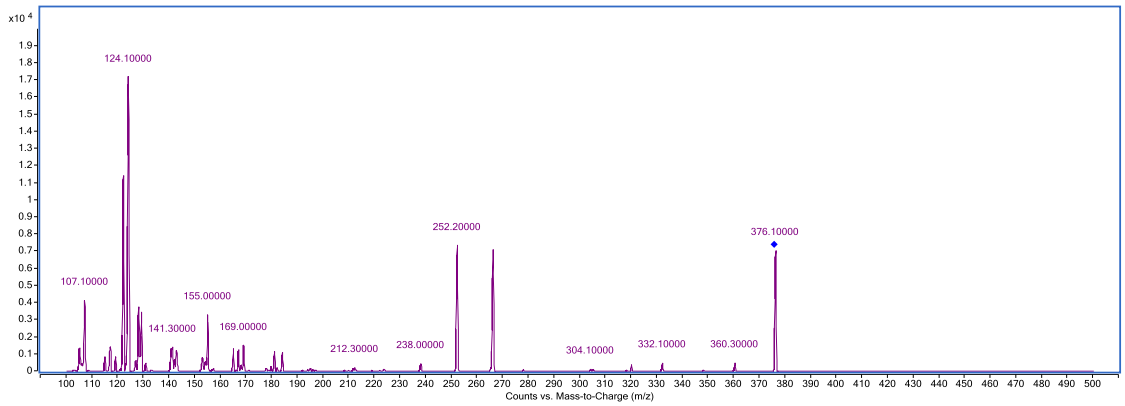


Figure 4.33 MS/MS spectrum for DMP derivative of estrone obtained on an Agilent QQQ instrument at 35 V with argon collision gas.

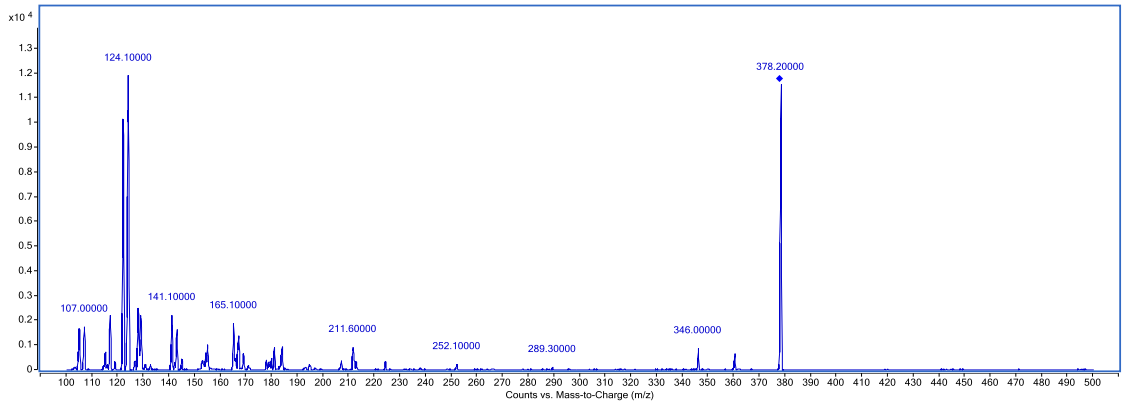


Figure 4.34 MS/MS spectrum for DMP derivative of estradiol obtained on an Agilent QQQ instrument at 35 V with argon collision gas.

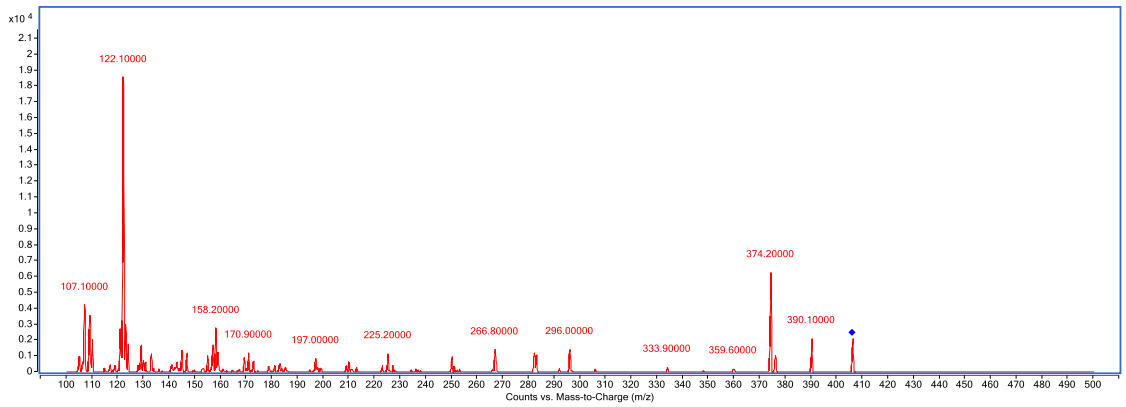


Figure 4.35 MS/MS spectrum for DMP derivative of methoxy estrone obtained on an Agilent QQQ instrument at 35 V with argon collision gas.

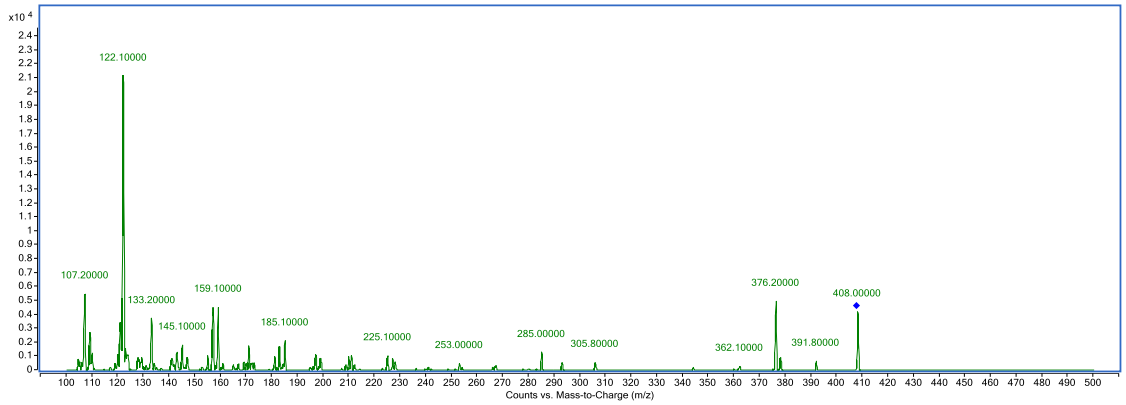


Figure 4.36 MS/MS spectrum for DMP derivative of methoxy estradiol obtained on an Agilent QQQ instrument at 35 V with argon collision gas.

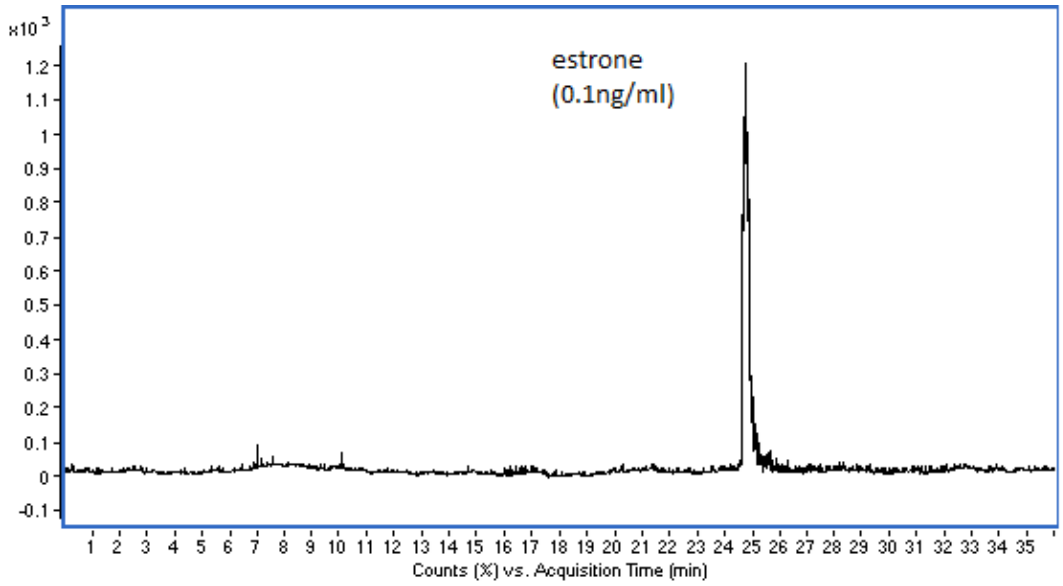


Figure 4.37 SRM trace for the DMP derivative of estrone at 0.1 ng/ml on the Agilent QQQ instrument monitoring the transition from m/z 376 to m/z 266.

Thus the tandem MS method requires more work to provide a suitable method for these derivatives and it was decided to continue with the Orbitrap method. The results suggested that the best resolution of estrogens and their metabolites and peak shapes was obtained by using C18-AR columns and a Thermo Orbitrap mass spectrometer. The estrone and estradiol and their metabolites were detected well in the standard mixture, the separation pattern and peak shapes was also good and the reliable limit of detection was *ca* 0.1 ng/ml. The method was then applied to the media samples provided by the University of Glasgow. These samples were prepared by incubating pulmonary artery cells with estradiol at a concentration of *ca* 15 ng/ml, thus the method should easily detect estradiol but the metabolites are formed in much smaller

amounts. In order to increase sensitivity 4 ml of incubation medium was extracted by using SPE on Strata X cartridges. After some preliminary runs an experimental run was achieved where it was possible to see some of the estradiol metabolites. The major conversion was into estrone and most of the estradiol appeared to be converted into estrone over 24h of incubation with the cells (figure 4.38). There was still some estradiol remaining after incubation (figure 4.39) and in one sample conversion to epi estradiol appeared to have taken place.

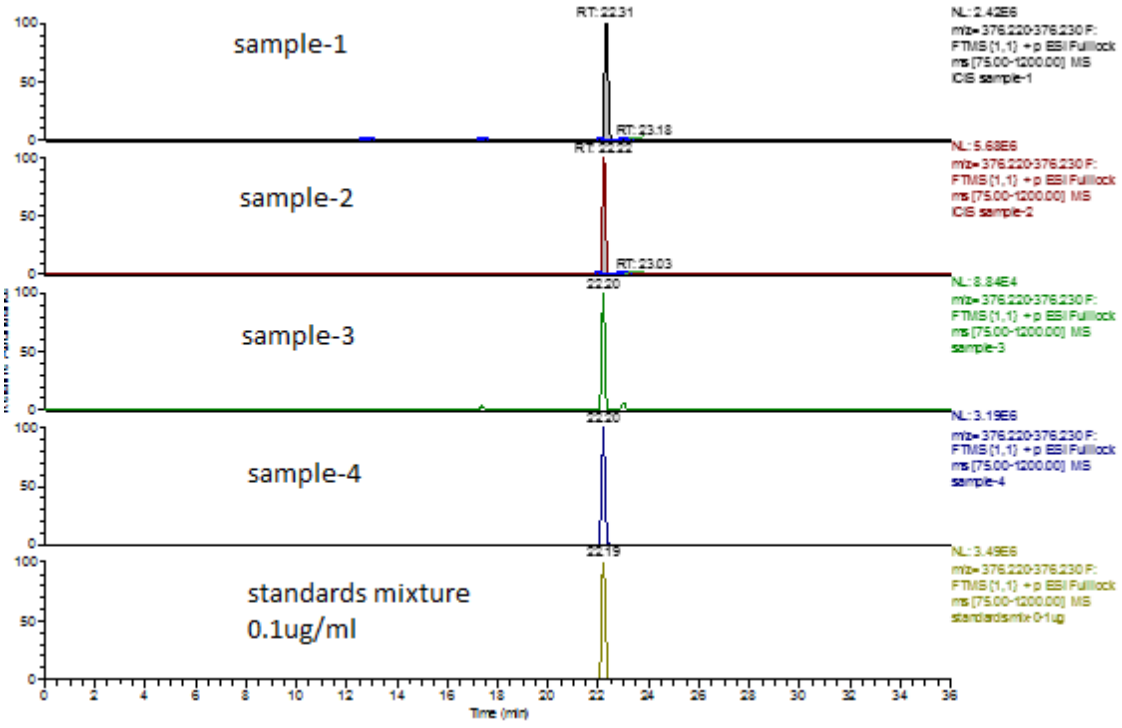


Figure 4.38 Estrone detected in all the media samples and standard mixture. The results obtained was very satisfactory because estrone was detected in both the media samples and standard solution as clear from the figure.

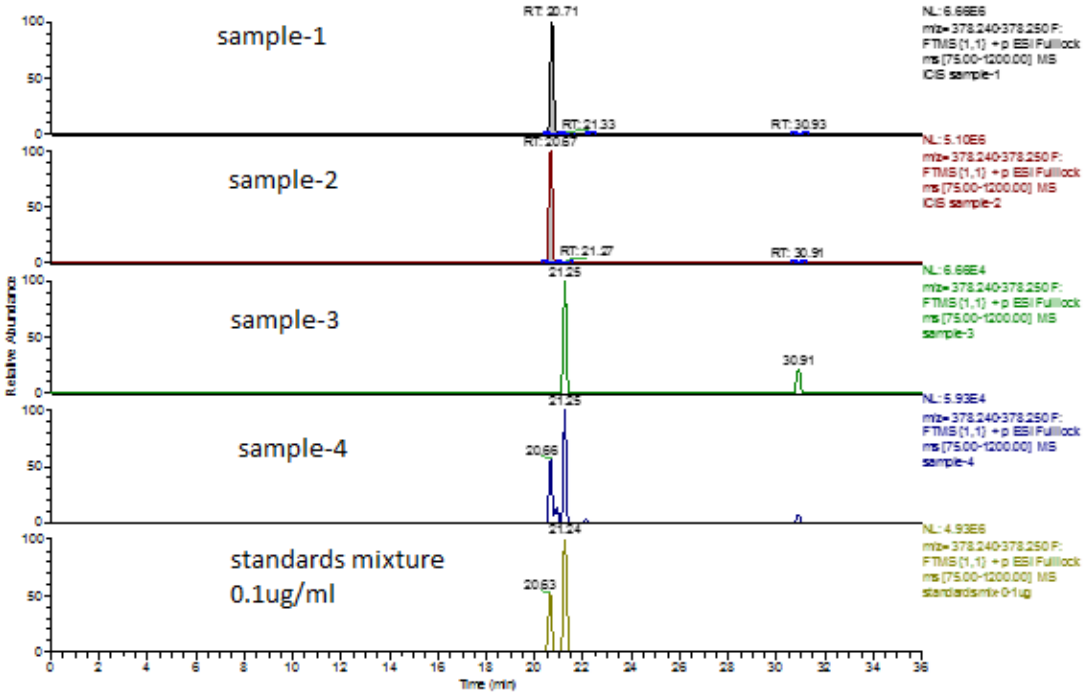


Figure 4.39 Estradiol and its epimer also detected in both media samples and standard mixture. The results obtained for estradiol was also very satisfactory because estrone was detected in both the media samples and standard solution as seen in the figure.

There was no indication of the presence of methoxy metabolites of estrone in the samples (figure 4.40). However, in some of the samples there were clear peaks for hydroxy estrone (figure 4.41). In one sample there was a peak matching methoxy estradiol (figure 4.42). Two samples contained a hydroxy estradiol metabolite (figure 4.43).

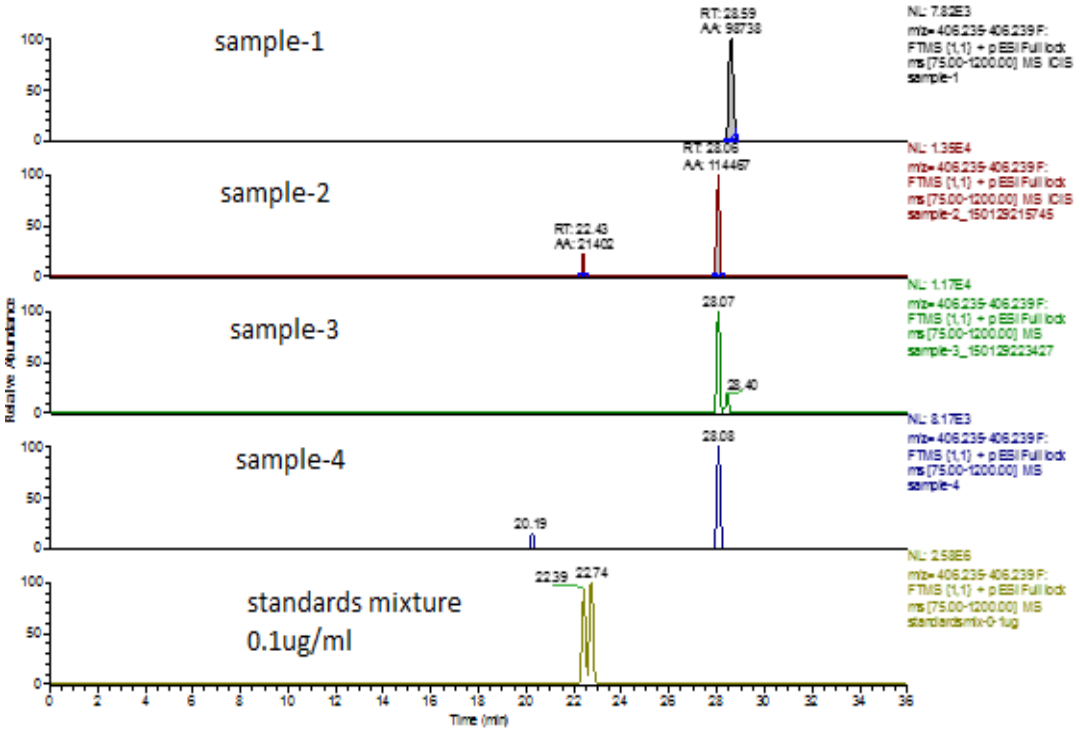


Figure 4.40 Hydroxy methyl metabolites of estrone. No metabolites detected. The peaks running later than the standard may be isomers although their masses deviate by about 3 ppm.

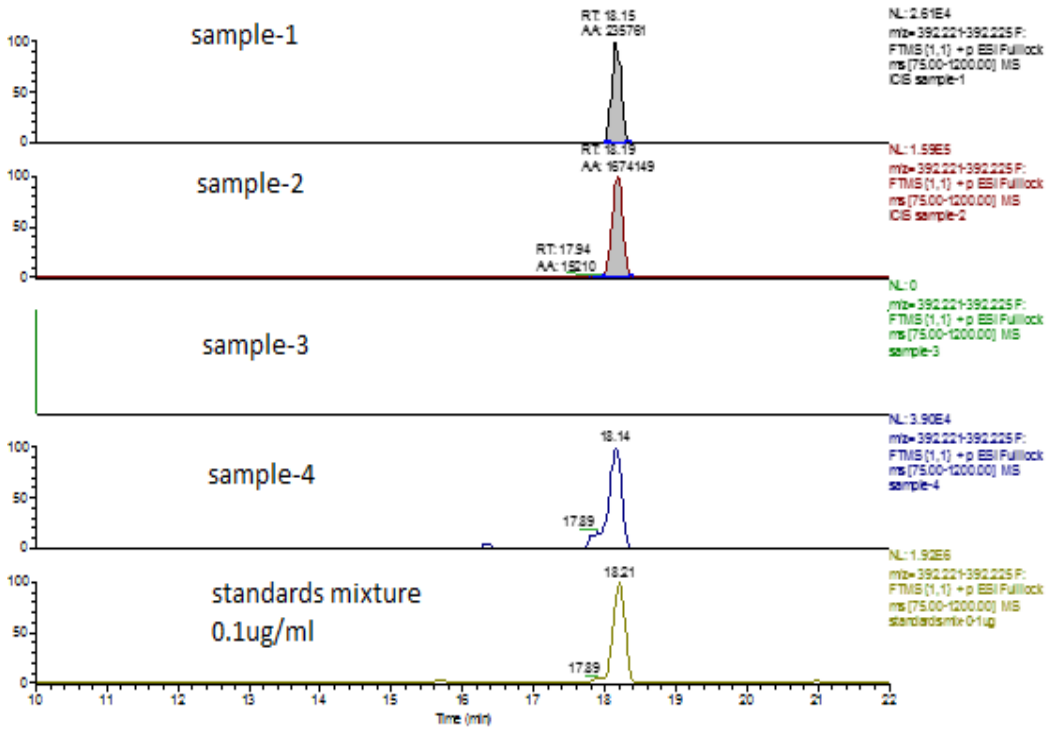


Figure 4.41 represents the metabolite hydroxy estrone in media samples and standards mixture. The results obtained was good because clear peaks in three of the samples matching the standard.

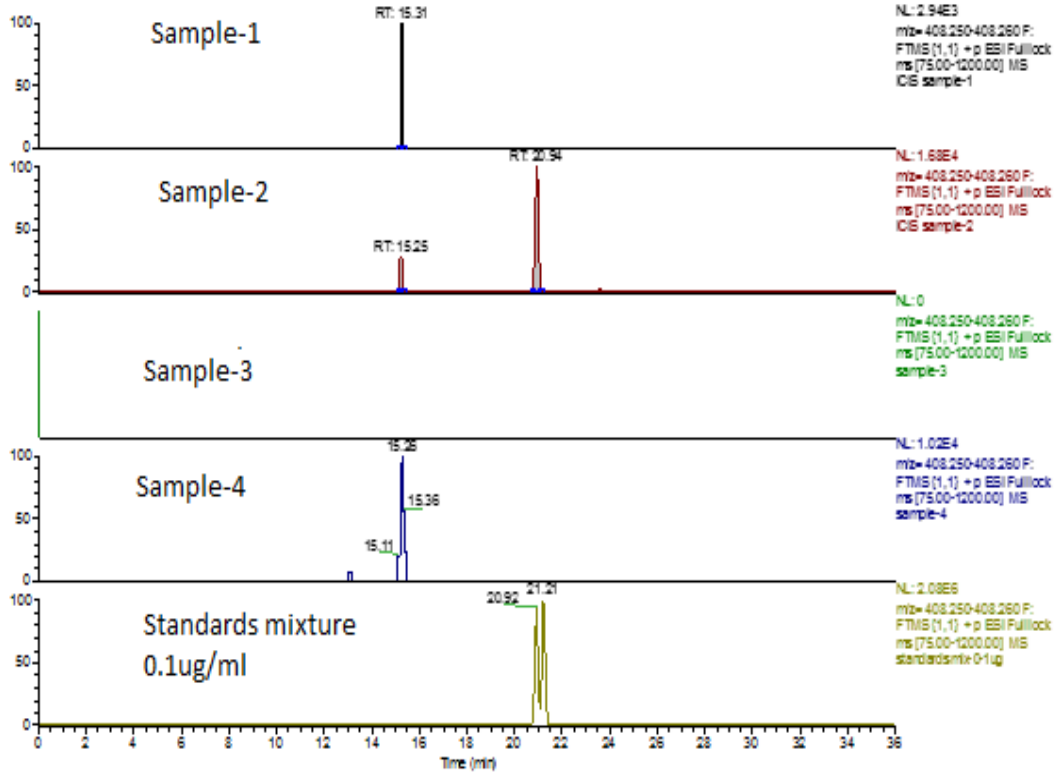


Figure 4.42 showing estradiol methoxy in four media samples and mixture of standard estrogen solution. Only sample 2 has a metabolite matching the standard.

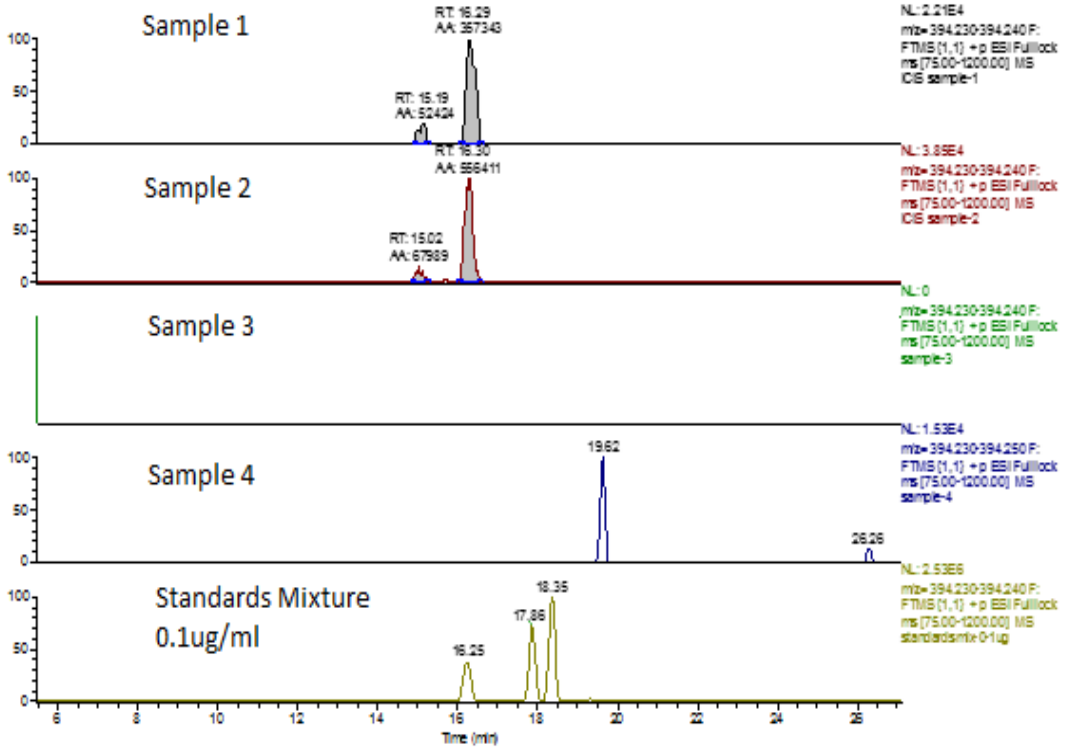


Figure 4.43 represents hydroxy estradiol in media samples and standard solution of steroids mixture. Samples 1 and 2 have metabolites matching one of the standards.

4.4 Conclusion

Initially we tried picolinic acid, trigonelline hydrochloride and (3-carboxy propyl) trimethyl ammonium chlorides as derivatization reagents, but the results obtained were not reliable and sensitive enough to be used for the derivatization of estrogens. Derivatisation with DMP works well for determination of estrogens and their metabolites and can be used for the quantification of these steroids in biological samples. The method is sufficiently sensitive to work with the Orbitrap for the

determination of estrogen metabolites in culture medium. It will be possible to transfer the method to a dedicated tandem MS system and possibly increase the limit of detection by 10-100 times. The method has been transferred to the student working on the cell culture models at the University of Glasgow and they have achieved similar limits of detection at *ca* 1ng/ml on the Orbitrap.

Chapter 5

5 Determination of Artemether and its metabolite Dihydroartemisinin (DHA) in human plasma by High performance Liquid Chromatography- Mass Spectrometry

5.1 Introduction

Malaria is the main cause of public health problems and is responsible for widespread morbidity and mortality in the developing countries of the world. According to a study more than 100 million cases of malaria are reported every year and more than 2 million people die every year because of malaria [133, 134]. The outcomes of the disease have become more serious due to the resistance developed by malaria parasites to the available antimalarial drugs like chloroquine, sulfadoxine, pyrimethamine, and mefloquine. However, there is a continuous struggle for the development of new potent drugs against the malaria parasite and it was the Chinese scientists who succeeded in the isolation and purification of a new drug artemisinin. This new drug has helped in the control of the disease resulting in the marked decrease in the mortality due to malaria seen in the last decade [135].

Malaria is an acute feverish disease caused by an organism called a haemoprotozoa belonging to the genus plasmodium [135, 136, 137, 138]. The disease usually occurs in

the tropical and semi tropical parts of the world. The disease is spread by the bite of the female anopheles mosquito [136, 138]. The history of malarial disease is very old and there has been a continuous struggle by mankind to fight the disease which has affected a large part of the human population. It has been known for a very long time that the malaria disease is associated with marshes or standing water. In the late 19th century Ross found that mosquitoes were involved in the spread of the disease and served as a vector while another scientist Charles Louis Alphonse Laveran reported the plasmodium to be the pathogen causing the disease [139]. There are different species of plasmodium which have been identified but only five species cause the disease in humans. These species are *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*) *Plasmodium malariae* (*P. malariae*), and *Plasmodium knowlesi (P. knowlesi)*. *Plasmodium falciparum* (*P. falciparum*) is considered to be the most pathogenic and to be responsible for most of the complications of the disease [109]. These species which cause disease in humans differ from each other in the periodicity of their life cycles and the consequences of the disease. The clinical symptoms of the disease generally include high fever, chills or shivering, prostration and anaemia. More severe cases of the disease result in metabolic acidosis, delirium, cerebral malaria and failure of multiple organs resulting in coma and death [137, 139].

5.1.1 Life cycle of malaria parasite

The life cycle of malaria parasite is a complex one and is completed in the mosquito and host human. It consists of an asexual stage and sexual stage. The asexual stage occurs in humans comprising of two stages, an asymptomatic liver stage followed by symptomatic blood stage and then the sexual stage which occurs in the female mosquito.

The liver stage is started by the bite of a female mosquito and sporozoites are introduced into the body of the host which then travel to the liver and infect the cells there. This stage is clinically silent and asymptomatic with thousands of merozoites produced after asexual multiplication. These merozoites are then released into the blood. The merozoites after entering the red blood cells infect them and go through asexual reproduction several times resulting in clinical illness. This stage is called erythrocyte stage of infection. Some merozoites in the blood stream develop into gametocytes which are taken up by the mosquito when it bites the infected human. The gametocytes then undergo sexual development in the mosquito to produce sporozoites which then travel to the salivary glands of the mosquito and again the sporozoites are transferred to another person through the bite [139].

5.1.2 General considerations

Artemether (figure 5.1) is a lipophilic drug and a methyl-ether derivative of artemisinin [140, 141]. It has a logP value of 3.5 [141].

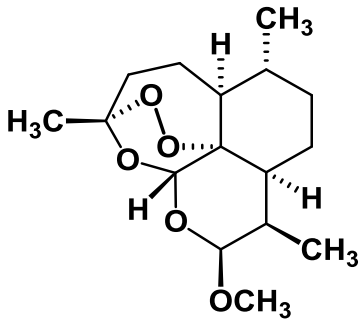
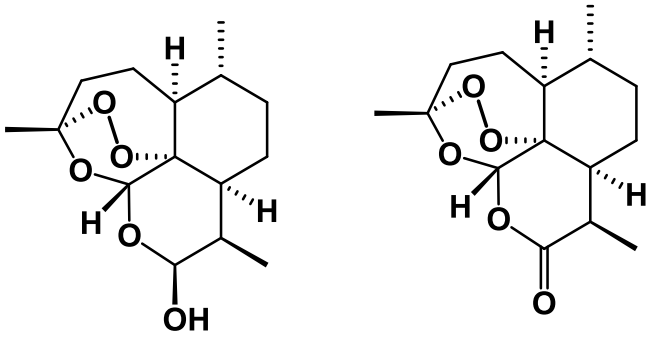


Figure 5.1 The structure of artemether

Artemisinin is also called Qinghaosu in Chinese [142] and was first obtained by Chinese scientists in 1972 from *Artemisia annua*. It is also called sweet or annual wormwood. The drug was obtained from the leaves and flowers of Qinghaosu and was used in China for over 2000 years for the treatment of feverish conditions [143].

Chemically artemisinin (figure 5.2) and its derivatives are sesquiterpene trioxane lactones having a peroxide bridge which is necessary for its antimalarial activity. The reduction of the lactone with sodium borohydride will produce dihydroartemisinin (figure 5.2) which also has high antimalarial activity [143, 144].



dihydroartemesinin artemesinin

Figure 5.2 Structure of dihydroartemisinin (DHA) and artemisinin

5.1.3 Mechanism of action

Artemether is thought to produce a very quick decline in parasitaemia after their administration by killing all the stages of the malarial parasites. The antimalarial action is due to its capability for producing free radicals. Artemether reacts with haeme (Fe^{2+}) which produces carbon centred free radicals. These free radicals are then responsible for alkylating proteins and causing damage to micro-organelles and rupturing the membranes of parasites thus killing them [143].

An alternative mechanism depending on the inhibition of the malaria parasite's sarcoplasmic reticulum (SR) calcium ATPase has also been suggested by some scientists [145].

5.1.4 Pharmacokinetics

Artemisinin is the parent drug of this class of compounds. Different derivatives are obtained by modifying the structure of artemisinin. The administration route for each

derivative of artemisinin is different depending on their physical properties and the dosage form of the derivative [146].

Artemisinin is formulated in oral and rectal dosage forms [140, 146]. Dihydroartemisinin (DHA) is the main metabolite of all derivatives of artemisinin and is responsible for their antimalarial action. However, the parent compound artemisinin is not metabolized to DHA and produces its own mode of action. The conversion rate of the artemisinin derivatives to DHA is different depending on the particular compound, and these compounds serve as prodrugs offering improved oral absorption. The derivatives also help in promoting their own metabolism following repeated dosing because of the auto-induction of CYP2B6. Artemisinin and its derivatives can easily pass the blood brain barrier (BBB) as well as the placenta. Protein binding of artemisinin ranges from 43 to 82% [143].

Artemether is available in oral, rectal and intramuscular preparations [140, 146]. Protein binding for artemether is 77% and the main protein binding the drug is α1- acid glycoprotein which binds about 33% of the drug. Albumin, high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) also contribute to the binding of the drug [146]. The absorption rate of artemether is fast and it is also rapidly cleared from the plasma. The elimination half life is short and is 2 to 3 hours [134]. Artemether is metabolized mainly by the Cytochrome P450 3A4 (CYP3A4) enzyme. There is a high inter subject variability for artemether metabolism in healthy individuals and patients [134]. Artemether is metabolized in the liver into its

main metabolite DHA which has higher antimalarial activity than the parent drug [140]. Artemether after being administered orally undergoes first pass metabolism and is converted to dihydroartemisinin (DHA). Artemether is distributed extensively in the body and its concentration ranges from 43 to 82% [147] with the highest concentration occurring in the brain [146]. The pharmacokinetic characteristics of artemether change with the route of administration. According to one study the bioavailability obtained after oral administration was higher than following intramuscular administration. It was also shown that the plasma concentration of artemether was higher during the phase of acute illness than during the period of recovery or convalescence [146].

5.1.5 Clinical applications

5.1.5.1 Uncomplicated malaria

Artemether have been used in a large number of patients for the treatment of malaria in south East Asia with no major side effects being reported. There was no significant difference found in the safety, efficacy and toxicity profiles among the different artemisinin derivatives and they all showed effectiveness against all malarial parasites. The drug showed good tolerability and was effective in removing the blood parasites in two days.

Artemether should be given in combination with another drug (lumefantrine) having effective antimalarial activity in order to prevent recrudescence and the development of resistance [143].

5.1.5.2 Severe complicated malaria

Severe malaria is responsible for the mortality of about 15 to 20% of hospitalised patients regardless of antimalarial drugs and proper care support. Due to increased resistance developed to chloroquine, severe malaria is treated by either cinchona alkaloids or the artemisinin class of drugs. In Europe and Africa quinine was used frequently in earlier times despite having a narrow therapeutic index and being responsible for hyperinsulinaemic hypoglycaemia which is more severe in pregnancy. It also affects the heart being responsible for the prolongation of the QT interval after parenteral administration. Intramuscular administration has been shown to be effective but it is also responsible for local toxicity and hypoglycaemia. In South East Asia there has been resistance developed to many drugs so artemether and other artemisinin derivatives are the only options left to treat severe malaria [145].

According to the World Health Organization the following dosing regime is recommended for adults and children over 6 months of age.

I/M artemether: on first day 3.2 mg/kg should be given as a loading dose which is then followed by a daily dose of 1.6 mg/kg for at least three days until the patient starts taking the oral antimalarials.

I/V artesunate: a loading dose of 2 to 2.4 mg/kg should be given as loading dose and then 1 to 1.2 mg/kg daily for at least three days until the patient start taking the oral antimalarials.

It is also necessary that general management should be considered for all patients with severe malaria including those with parasitological confirmation and in intensive care and control of body fluids and blood glucose level, coma treatment and urine output should be monitored [143, 148].

5.1.6 Side effects of artemether

Artemether has reported to cause neurotoxicity and cardiotoxicity in experimental animals however the possibility of these adverse effects in humans is much less. The most common adverse effects caused by the drug are nausea, vomiting, dizziness and anorexia which are common in acute malaria, severe allergic reaction in rare cases and haemolysis. Cardiotoxic effects produced by these drugs are only shown as the prolongation of QT interval [143, 145]. Studies on animals showed that artemisinin and its derivatives are not mutagenic. Overall artemisinin and its derivatives are very safe and effective antimalarials having minimal side effects [143].

5.1.7 Other problems

5.1.7.1 Recrudescence

According to the literature artemether are very effective against plasmodia of all types particularly *Plasmodium falciparum* and *Plasmodium vivax*. The recovery rate with artemisinin and its derivatives is very high as compared to other antimalarial drugs [143]. Recrudescence is common with artemether when it is used in monotherapy unless it is used in high doses for several days. Its combination with lumefantrine

makes it a drug of choice for malaria [134]. The recrudescence rate varies from 44 to 54% when artemether are used for 3 days, however the cure rate increased to over 90% with treatment of up to 7 days [143].

5.1.7.2 Pregnancy

Artememther is not recommended to use in the first trimester of pregnancy for the treatment of uncomplicated malaria however it can be given in the 2nd and 3rd trimester of pregnancy. In severe malaria they can also be used in 2nd and 3rd trimester of pregnancy. In the 1st trimester there is little data available about its use. However, it has the advantage of lower risk of hypoglycaemia as compare to quinine in the 1st trimester of pregnancy.

5.1.7.3 Prophylaxis

There is no data available of using artemether for prophylaxis of malaria they are only used in the treatment of malaria [143].

5.1.8 Determination of artemether and its metabolite DHA by LC-MS

There are a number of methods reported for the quantification of artemether and its metabolite dihydroartemisinin (DHA) [137, 149]. The techniques used for the determination of antimalarials include thin layer chromatography, gas chromatography, spectroscopic and immunological methods, the methods most commonly used are high performance liquid chromatography coupled with either ultra

violet detection, evaporative light scattering detection, electron capture detection or electrospray ionisation mass spectrometry detection [148].

Numerous methods have been described for the determination of malaria drugs in different biological samples. However HPLC coupled with mass spectrometry is the most preferred technique recently. The LCMS methods used to determine antimalarials in different biological matrices has been reviewed [150]. These antimalarial drugs have been detected in different biological samples including plasma, urine etc.

Techniques which are generally used for the preparation of samples are liquid-liquid extraction (LLE), solid phase extraction (SPT) and protein precipitation (PPT). PPT is a cheaper method however SPE is considered as a cleaner method and employed where higher sensitivity is required because the sample is concentrated following extraction. LLE combines these two techniques so it is the most widely used method. But SPE and LLE are thought to be the time consuming techniques requiring high volume of non-polar solvent.

Reversed phase columns are most commonly employed for the separation and detection of all antimalarial drugs with over 70% of the methods using C18 columns for the detection of the drugs.

Therapeutic drug monitoring is necessary for malaria drugs having narrow therapeutic windows and dose dependent unwanted effects. Therefore it is necessary to determine the concentration of these drugs in biological samples. So a very accurate, reliable,

sensitive as well as reproducible method is required for the determination of anti-malarial drugs. In most of the bioanalytical liquid chromatographic methods, the preferred detection is MS/MS which is the most sensitive analytical tool used for the quantification of a number of compounds simultaneously in one single analysis. Also the use of silanised glass tubes is recommended for the extraction of these compounds to avoid the sticking of these compounds to the glass walls of sample vials [150].

5.1.9 Aim of the study

The main aim of this study is to determine artemether and its metabolite DHA in human plasma by HPLC-MS. The method will then be used to determine the drug in plasma of healthy volunteers who have received fixed dose combination tablets.

5.2 Experimental

5.2.1 Chemicals and solvents

Artemether, $C_{16}H_{26}O_5$ Mol. Wt 298.38, DHA $C_{15}H_{24}O_5$ Mol. Wt 284.35, internal standard artemisinin $C_{15}H_{22}O_5$ Mol. Wt 282.33, Dichloromethane and *tert*. Methyl butyl ether was obtained from sigma Aldrich. HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. AnalaR grade formic acid (98%) was obtained from BDH-Merck, UK. Drug free human plasma was provided by the Golden jubilee hospital, Glasgow and was stored at -32^{o}C.

5.2.2 Preparation of standards

Primary stock solutions for artemether, DHA and internal standard artemesinin were prepared by dissolving 1 mg in 1ml of acetonitrile. Then different concentrations of working solutions were prepared from the stock solution in acetonitrile.

5.2.3 Extraction procedure

The spiked plasma was extracted by the following method:

To 0.5ml of plasma add 1ml of a mixture of dichloromethane and *tert*.-Methyl butyl ether (8:2 v/v). The mixture was then vortexed for 30 seconds before centrifuging for five minutes at 10,000 rpm. The organic layer was then taken in to an HPLC vial and inserted into the HPLC-MS system for analysis.

5.2.4 Preparation of plasma samples

Dried extracts from plasma samples taken from volunteers for this study were provided by University of Peshawar Pakistan, with the aim of studying the pharmacokinetic interaction of artemether with pomegranate juice. Twenty six healthy human volunteers participated in the study after ethical approval. The participants were advised not to take any medication, alcoholic products, caffeine and any type of grape fruit juices for a period of at least 7 days before and during the study period. This was a randomised open label, single dose cross over study with a washout time of 14 days where the volunteers were randomly divided into two groups, each group of 13 volunteers. During the first stage of the study the volunteers of group 1 was given fresh

pomegranate juice 2 times a day for a week. On day 7 the morning after overnight fasting a single oral dose of Coartem was given with 250 ml of pomegranate juice while the volunteers of the second group took the tested drug with water. The order was then reversed for the second study period. After intake of the drug the volunteers were given a standard breakfast and meal after 4 and 10 hours of the drug intake respectively. The blood samples were taken from the volunteers for the analysis of artemether and its metabolite DHA in the plasma at predose (0h) and then at different time intervals of 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 hours after administration of Coartem. After collection from the volunteers the plasma was then separated from blood by centrifugation and was stored at -80^{o}C before being sent to the University of Strathclyde for analysis.

5.2.5 Preparation of spiked plasma

The blank plasma was spiked with IS (500ng) and varying concentrations of artemether and DHA. The samples were then extracted with the method mentioned above before injecting into the LC-MS.

5.2.6 Liquid chromatography - mass spectrometry analysis

Measurement of standards and samples was carried out isocratically on a Surveyor HPLC system combined with an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). An ACE 5 C18-PFP COLUMN (150 × 4.6mm, 5µm Hichrom, Reading, UK) fitted with a guard column having the same packing material was used. Mobile phase A consisted of 0.1% v/v formic acid in water and Mobile

phase B consisted of acetonitrile only and isocratic elution was carried out with A:B 20:80, v/v. The flow rate was 500µl/min. The ESI interface was operated in a positive ion mode with a spray voltage of 4.5 kV. The temperature of the ion transfer capillary was 275°C and the flow rates of the sheath and auxiliary gases were 50 and 17 arbitrary units respectively. The full scan range was m/z 75 to 1200. The data was recorded using Xcalibur 2.1.0 software (Thermo Fisher Scientific). Mass calibration was performed for both ESI polarities before the analysis using the standard Thermo Calmix solution and the signals at 83.0604 m/z (2xACN+H) was selected as a lock masses for positive ion mode during each analytical run.

5.3 Results and discussion

In developing a method for the determination of artemether and its metabolite DHA in plasma various parameters were tried. Initially we used ammonium acetate buffer in the mobile phase in order to promote formation of the ammonium adduct of artemether according to the literature [151], but there was no clear peak observed for the ammonium adduct of artemether or DHA. Then 0.1% FA in acetonitrile and 0.1% FA in water were evaluated in different ratios, and the results still were still not good. There was also tailing observed in the peaks detected for artemether which may be due to the degradation of the compound at low *p*H. Then we tried 0.1% FA in water and acetonitrile only in different ratios and the best results were obtained with 20:80 of 0.1% FA in H2O: ACN. The addition of formic acid to the mobile phase helps to improve the peak shape and promote the ionization of a compound in positive mode.

Thus the method was reasonably robust although these analytes are difficult to analyse under ESI conditions since there is no particular centre which can be readily protonated. The chromatographic and ESI mass spectra of artemether, DHA and the internal standard artemisinin in a standard solution and some plasma samples are shown in figures 5.3-5.6. Artemether is extensively metabolized to its metabolite dihydroartemisinin (DHA) in liver which possess a stronger antimalarial activity than the parent drug [152]. As artemether is not a very biologically stable compound and was immediately metabolized to its active metabolite DHA, no peak was observed for artemether. So we therefore considered DHA as the active metabolite of artemether and to be the main compound of interest which was to be quantified. Both artemether and DHA after ionization undergo neutral losses of methanol and water respectively in the source of the mass spectrometer and these were the ions which were used to quantify the compounds. The internal standard artemisinin was stable and the protonated molecule was detected at m/z 283. The ions observed for the other two compounds were (MH^+-CH_3OH) m/z 267.15 for artemether, (MH^+-H_2O) m/z 267.15 for DHA and (MH^+) m/z 283.15 for internal standard artemisinin. The observed retention times were about 7.1 min for artemether, 4.1 min for DHA and about 5 min for the internal standard artemisinin.

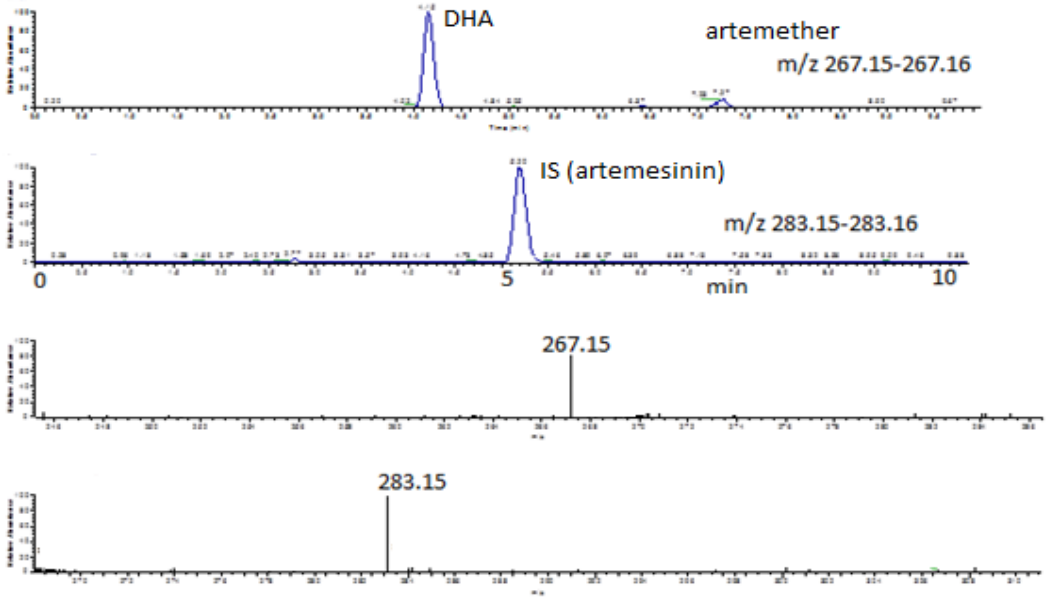


Figure 5.3 Extracted ion chromatograms and mass scan spectra of artemether, DHA (1 µg/ml) and IS (0.5 µg/ml) in standard solution.

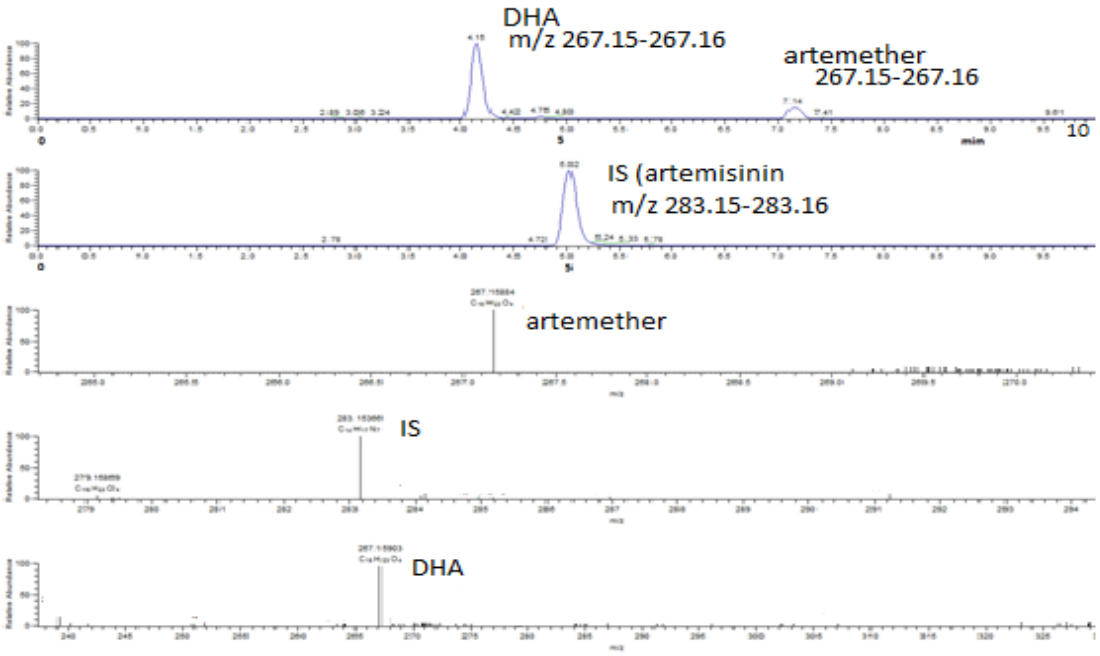


Figure 5.4 Extracted ion chromatograms and mass scan spectra of artemether, DHA (3.2 µg/ml) and IS (0.5 µg/ml) in standard solution.

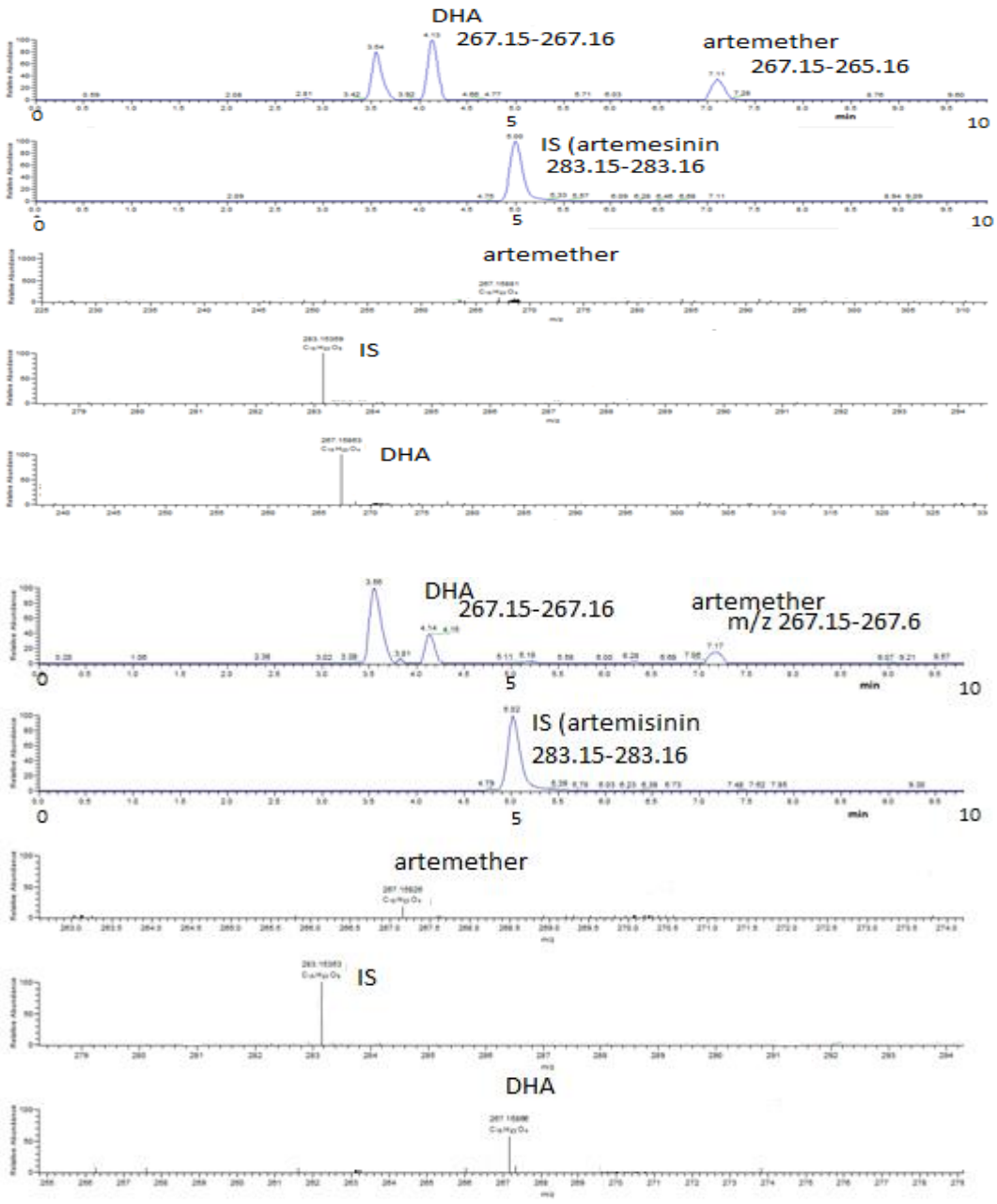


Figure 5.5 Extracted ion chromatograms and mass spectra of artemether, DHA and IS extracted from plasma obtained from volunteers. Volunteers were given a fixed dose of Coartem tablet having the concentration of artemether of 20mg according to packing specification. The concentration of IS is 0.5 µg/ml.

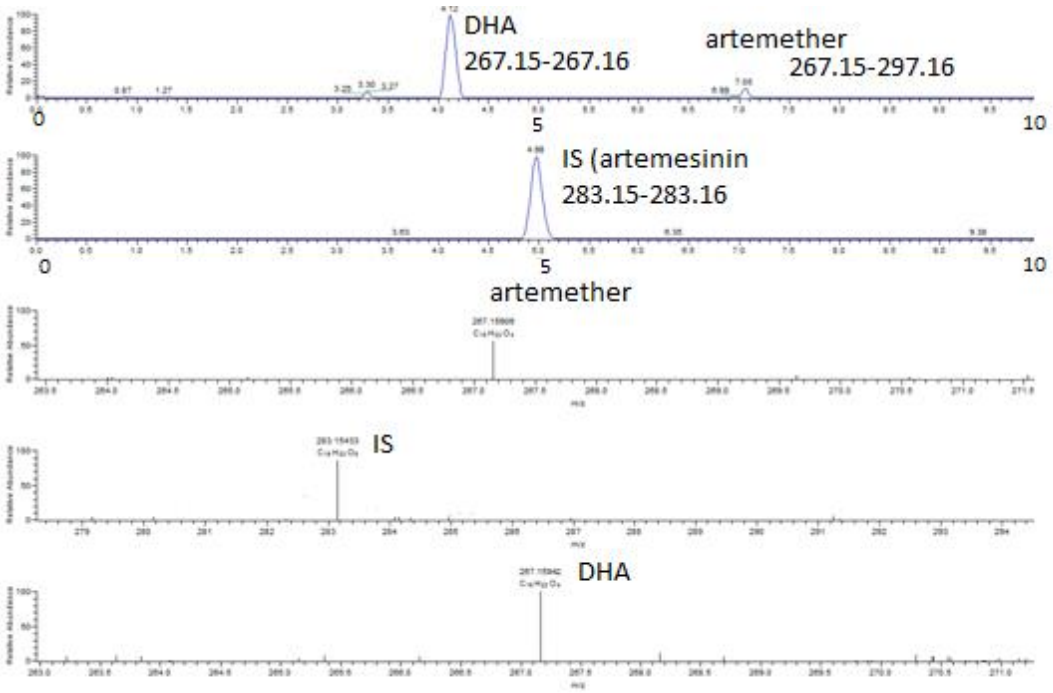


Figure 5.6 Extracted ion chromatograms and mass scan spectra of artemether, DHA and IS in spiked plasma. The concentration of artemether in the spiked plasma was 1 µg/ml while the concentration of IS artemisinin was 0.5 µg/ml. The chromatohraphic conditions used to obtain the results for figure 5.3 to 5.6 are: column C-18 PFP, mobile phase A 0.1% FA in water and mobile phase B was acetonitrile used in 20:80 with a flow rate of 0.5 ml/min.

There were two peaks observed in the plasma samples obtained from volunteers which according to literature [153] are due to α- and β- tautomers of DHA. The formula corresponding to the artemether peak at m/z 267 was $C_{15}H_{23}O_4$ after the loss of CH_3OH and the formula for DHA was $C_{15}H_{23}O_4$ at m/z 267 after the neutral loss of an H_2O molecule from DHA at 5ppm. The IS has the formula of $C_{15}H_{23}O_5$ corresponding to the peak at m/z 283.

5.3.1 Calibration

The ion at m/z 267 was the most suitable for quantification of dihydroartemisinin (DHA) and a calibration curve was prepared. Solutions of varying amounts of DHA and a fixed amount of the IS artemisinin were prepared in acetonitrile ranging from 0.1 µg/ml to 3.2 µg/ml and were injected to the HPLC-MS system. Concentration of DHA in micrograms was then plotted against the peak area ratio of DHA/IS, and the response was reasonably linear (R2= 0.985). The equation of the calibration plot was y = 0.166x + 0.015. The calibration line is shown in figure 5.7.

Table 5.1 Results of calibration curve of DHA

CONC(µg/ml)	DHA	IS(Artemesinin)	Ratio
0.1	201403	13452618	0.014971
0.2	344023	11145521	0.030866
0.4	1962754	22833748	0.085958
0.8	1429450	8919842	0.160255
1.6	6291123	19451879	0.32342
3.2	8276801	15775990	0.524645

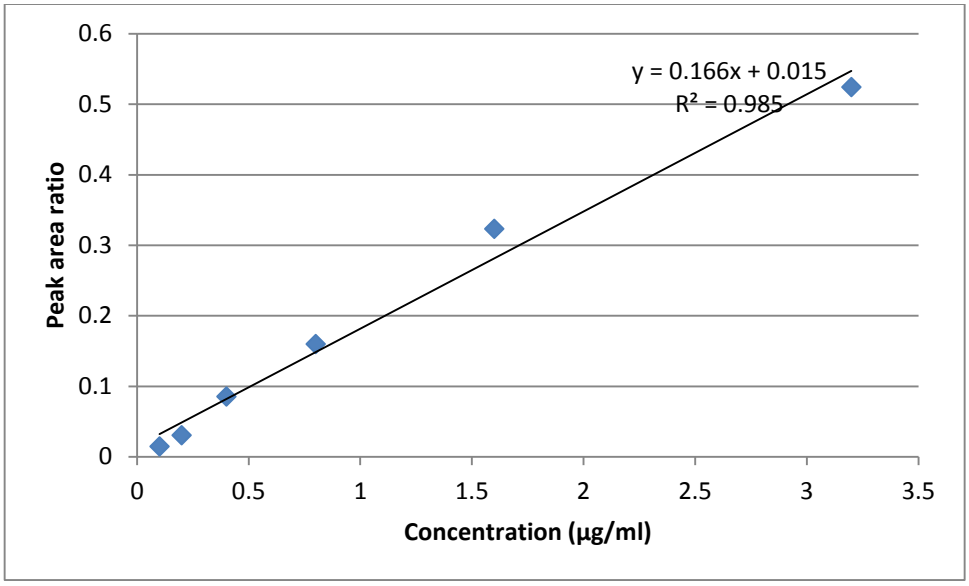


Figure 5.7 Calibration curve for DHA in the range 0.1 – 3.2 µg/ml.

5.3.2 Analysis of spiked plasma

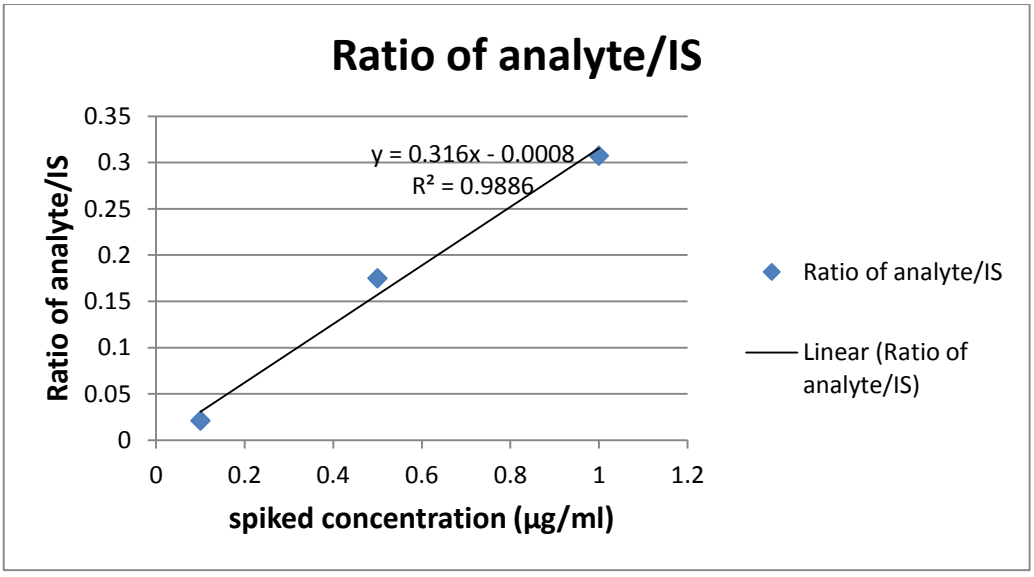
Blank plasma was spiked with three different concentrations of artemether and DHA and extracted with the method described above (section 5.2.3). It was then injected into the mass spectrometry system for analysis. The observed peaks for artemether and DHA were similar to those of standards and patient plasma samples, and the formulae were confirmed by Xcalibur software.

5.3.3 Analysis of clinical samples

The clinical samples for this study were provided by Department of Pharmacy the University of Peshawar, Pakistan. Extraction was carried out in Pakistan according to the protocol described in section 5.2.3 and the samples were dried down and sent to Glasgow. The study was performed involving 26 volunteers who were given a single

dose of Coartem®. Then plasma samples were taken from the volunteers at different time intervals. The plasma was then separated from the blood cells and stored at -80^{o}C until extraction. The analysis of plasma samples from the patients or volunteers showed the same peaks for artemether and DHA as were observed in the standard solutions or in spiked plasma. The concentration of drug in each sample was quantified from the peak area of the samples.The data is shown below in the table.

Formulations of artemether are generally well tolerated with no significant adverse effects. However there is a significant inter-individual variability in the pharmacokinetic parameters of artemether and DHA. According to literature the artemether is detected after *ca* 30 minutes in most cases and reaches the maximum plasma concentration of *ca* 400 ng/ml in about 2 hours after which there is a decline in plasma concentration [125].



Three points calibration curve of spiked concentration vs ratio of analyte/IS

Determination of Dihydroartemisinin (DHA) in plasma samples obtained from patients

Patient samples	peak area of analyte	peak area of IS	Ratio analyte/IS	amount recovered in µg/ml
12a	862432	13984975	0.0617	0.1976
10b	736174	18120445	0.0406	0.1311
11b	817941	28307645	0.0289	0.0940
12b	666156	22149952	0.0301	0.0977
1c	637314	12115225	0.0526	0.1690
12d	646263	26056057	0.0248	0.0810
1f	157585	16409615	0.0096	0.0329
2f	842187	12649949	0.0666	0.2132
6f	2528682	25273006	0.1001	0.3192
9f	1470564	22836130	0.0644	0.2063
3g	946321	13995616	0.0676	0.2165
4g	1425418	15009947	0.0950	0.3031
9g	2018582	19332794	0.1044	0.3330
10g	656155	10542953	0.0622	0.1995
10j	2466633	39147534	0.0630	0.2019
11j	776784	9370776	0.0829	0.2649
10k	1153737	11642900	0.0991	0.3161
11k	1324783	12982793	0.1020	0.3254
1m	553723	16205888	0.0342	0.1107

11m	619490	8967698	0.0691	0.2211
1n	560957	6658888	0.0842	0.2691
2n	967524	15713362	0.0616	0.1974
6c	6036023	53381515	0.1131	0.3604
7c	708033	40408636	0.0175	0.0580
8c	812141	43880825	0.0185	0.0611
9c	1635578	41042311	0.0399	0.1286
10c	1378106	36828004	0.0374	0.1209
11c	2163816	23229316	0.0932	0.2973
1d	214138	35591009	0.0060	0.0216
2d	679100	34915259	0.0194	0.0641
3d	2061604	27125339	0.0760	0.2430
10e	36061	1149525	0.0314	0.1018
11e	993158	875527	1.1344	3.5923
12e	100393	8911862	0.0113	0.0382
2k	592621	6263847	0.0946	0.3019
3k	593346	5317697	0.1116	0.3556
10m	458256	21769462	0.0211	0.0691
11m	619490	8967698	0.0691	0.2211
1n	560957	6658888	0.0842	0.2691
2n	967524	15713362	0.0616	0.1974
7n	2264029	27879940	0.0812	0.2595

9p	2940977	39402447	0.0746	0.2387
12z	783648	12902934	0.0607	0.1947

5.3.4 Conclusion

A method for the analysis of DHA in plasma was successfully developed and was adequately sensitive for its quantification in plasma. Further validation of the method needs to be carried out with regard to precision and the stability of the analytes to storage in plasma.

6 Summary/Conclusion of the thesis

The summary or conclusion of the thesis is that it covers four projects, the details of which are described here briefly as:

The first chapter is the general introduction about the instruments and sample preparation methods used in the project.

The second chapter of the thesis describes the development of a method to measure free and bound ropivacaine in human plasma using equilibrium dialysis and HILIC chromatography coupled with a high resolution mass spectrometry. In this chapter a highly sensitive method for the determination of the free and bound portion of ropivacaine in patients undergoing knee and hip joint surgery was developed. Patient samples were provided by the Golden Jubilee Hospital, Clydebank. The method was validated according to FDA guidelines. The method is already published in Talanta journal. This method was used to analyse about 200 samples from hip joint surgery patients and about 250 samples from 50 knee joint surgery patients in two batches. The method was completely safe and in no case the drug reaches the toxic level. The clinical papers in collaboration were published in anaesthesia by the colleagues from Golden Jubilee hospital Glasgow based on the results determined by the method we developed in university of Strathclyde.

The third chapter in the thesis describes the determination of α1-glycoprotein in the plasma of patients undergoing knee and hip joint surgeries using an already established method using HPLC with a polymeric reversed phase column, thus enabling comparison

of the concentration of AGP protein with the levels of bound and unbound ropivacaine. This complete set of data should allow pharmacokinetic modelling studies to be carried out.

The fourth chapter covers the determination of the steroids estradiol and estrone and their hydroxyl and methoxy metabolites following a derivatization reaction for provision of improved sensitivity. The free steroids are not readily ionized by ESI mass spectrometry and derivatization procedure produces positively charged ions in order to enhance the detection of these steroids. Different reagents were used and the best sensitivity was obtained by using 2-fluoro 1, 3-dimethylpyridinium *p*-toluene sulphonate as the derivatising agent. This method was applied to samples obtained from patients with pulmonary hypertension in order to evaluate the role of steroids in the progression of disease. The results obtained from the derivatization is very satisfactory and detected very low level of estrogens in the samples provided by the university of Glasgow, however the plan is to use more sensitive mass spectrometer instruments using the already developed derivatization procedure in order to get further low levels of estrogens.

The last chapter of the thesis is about the determination of an anti-malarial drug artemether and its active metabolite DHA in human plasma using a reversed phase C18 column coupled with high resolution mass spectrometry, the samples were provided by the University of Peshawar, Pakistan. The method was developed for detection of this anti malarial drug and was then applied to the human plasma samples, the results

showed that the method is working well for the detection of artemether and its active metabolite DHA in human plasma, the method will be published in near future in appropriate journal.

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Appendix 1

Ropivacaine calculation sheet showing the concentration of ropivacaine in the plasma and buffer compartments for each sample

Knee joint surgery patients

- Batch 1 = 20 patients
- Batch 2 = 30 patients

Hip joint surgery patient

- Batch 1 = 20 patients

Key to the table in appendix 1 and example below showing determination of ropivacaine concentration in plasma and buffer chambers in the red device inserts and free and bound portion of ropivacaine

- A: Peak area of ropivacaine in sample
- B: Peak area of internal standard (lidocaine) in sample
- C: Peak area of internal standard in standard solution
- D: Peak area of ropivacaine in in standard solution
- Conc.: Concentration of of ropivacaine in the standard solution in µg/ml
- DF: Dilution factor

$$C = \frac{A}{B} \times \frac{C}{D} \times conc \times DF$$

In plasma

$$C = \frac{100685811}{176141120} * \frac{147607721}{1078905306} * 1 * 10 = 0.782$$

In buffer

$$C = \frac{2187479}{164016849} * \frac{147607721}{1078905306} * 1 * 10 = 0.0182$$

$$\text{Free \%} = \frac{\text{Conc. in buffer chamber}}{\text{Conc. in plasma chamber}} * 100$$

$$\text{Free \%} = \frac{0.0182}{0.782} * 100 = 2.333\ \%$$

Bound % = 100 - Free %

Bound % = 100 - 2.333 = 97.67%

Knee joint surgery patients
Batch 1

Patient-1											
Time	A	B	C	D	Chambers	Conc.in Buffer Chamber	Conc.in Plasma Chamber	100	% free	100	%Bound
						µg/ml	µg/ml				
Baseline	0	208108293	147607721	1078905306	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	170696609	147607721	1078905306	**Buffer**	0.000					
5 mins	100685811	176141120	147607721	1078905306	**Plasma**		0.782	100	2.333	100	97.67
5mins	2187479	164016849	147607721	1078905306	**Buffer**	0.018					
10 mins	137422357	180030042	147607721	1078905306	**Plasma**		1.044	100	1.766	100	98.23
10 mins	2101200	155910337	147607721	1078905306	**Buffer**	0.018					
15 mins	125132724	183820138	147607721	1078905306	**Plasma**		0.931	100	2.260	100	97.74
15 mins	2512143	163317345	147607721	1078905306	**Buffer**	0.021					
20 mins	115161488	183160859	147607721	1078905306	**Plasma**		0.860	100	2.280	100	97.72
20 mins	2317490	161673679	147607721	1078905306	**Buffer**	0.020					
25 mins	119733207	190697609	147607721	1078905306	**Plasma**		0.859	100	1.878	100	98.12
25 mins	1999582	169597206	147607721	1078905306	**Buffer**	0.016					
30 mins	113590106	184052936	147607721	1078905306	**Plasma**		0.844	100	1.792	100	98.21
30 mins	1730629	156453558	147607721	1078905306	**Buffer**	0.015					

1 hrs	109130459	178070032	147607721	1078905306	**Plasma**		0.838	100	1.688	100	98.31
1 hrs	1656768	160112010	147607721	1078905306	**Buffer**	0.014					
4 hrs	113597018	207981128	147607721	1078905306	**Plasma**		0.747	100	1.120	100	98.88
4 hrs	1031515	168672996	147607721	1078905306	**Buffer**	0.008					
24 hrs	118636217	168932835	147607721	1078905306	**Plasma**		0.961	100	1.037	100	98.96
24 hrs	1136589	156069744	147607721	1078905306	**Buffer**	0.010					
Pre-topup-1	109896804	167352934	147607721	1078905306	**Plasma**		0.898	100	1.322	100	98.68
Pre-topup-1	1409514	162373104	147607721	1078905306	**Buffer**	0.012					
Pre-topup-2	130725923	168156958	147607721	1078905306	**Plasma**		1.064	100	1.315	100	98.69
Pre-topup-2	1636954	160142582	147607721	1078905306	**Buffer**	0.014					
Pre-topup-3	94907647	131405731	147607721	1078905306	**Plasma**		0.988	100	1.398	100	98.60
Pre-topup-3	1382130	136842826	147607721	1078905306	**Buffer**	0.014					
Post-topup-2	136065696	168611195	147607721	1078905306	**Plasma**		1.104	100	1.320	100	98.68
Post-topup-2	1766847	165865449	147607721	1078905306	**Buffer**	0.015					
Post-	104078604	169369521	147607721	1078905306	**Plasma**		0.841	100	1.680	100	98.32

topup-3											
Post-topup-3	1658780	160641706	147607721	1078905306	**Buffer**	0.014					

Patient-2											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
						µg/ml	µg/ml				
Baseline	0	161203499	168787679	825702227	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	168883710	168787679	825702227	**Buffer**	0.000					
5 mins	103915658	174656857	168787679	825702227	**Plasma**		1.216	100	4.057	100	95.94
5mins	3947116	163537442	168787679	825702227	**Buffer**	0.049					
10 mins	120716593	181324104	168787679	825702227	**Plasma**		1.361	100	4.465	100	95.53
10 mins	4739308	159418512	168787679	825702227	**Buffer**	0.061					
15 mins	110212381	176552982	168787679	825702227	**Plasma**		1.276	100	3.785	100	96.21
15 mins	3951146	167219641	168787679	825702227	**Buffer**	0.048					
20 mins	100667308	174123792	168787679	825702227	**Plasma**		1.182	100	3.686	100	96.31
20 mins	3599885	168907346	168787679	825702227	**Buffer**	0.044					
25 mins	91552577	177979156	168787679	825702227	**Plasma**		1.052	100	3.592	100	96.41
25 mins	2989931	161823285	168787679	825702227	**Buffer**	0.038					

30 mins	87835858	176678369	168787679	825702227	**Plasma**		1.016	100	3.934	100	96.07
30 mins	3227680	165013804	168787679	825702227	**Buffer**	0.040					
1 hrs	76961995	180020369	168787679	825702227	**Plasma**		0.874	100	3.491	100	96.51
1 hrs	2354513	157754572	168787679	825702227	**Buffer**	0.031					
4 hrs	47998520	166416977	168787679	825702227	**Plasma**		0.590	100	3.804	100	96.20
4 hrs	1742336	158801912	168787679	825702227	**Buffer**	0.022					
24 hrs	64020098	165336652	168787679	825702227	**Plasma**		0.792	100	2.789	100	97.21
24 hrs	1776404	164500572	168787679	825702227	**Buffer**	0.022					
Pre-topup-1	55072321	169404178	168787679	825702227	**Plasma**		0.665	100	3.333	100	96.67
Pre-topup-1	1791737	165337766	168787679	825702227	**Buffer**	0.022					
Pre-topup-2	61998524	170484627	168787679	825702227	**Plasma**		0.743	100	3.485	100	96.52
Pre-topup-2	2159891	170402903	168787679	825702227	**Buffer**	0.026					
Pre-topup-3	58005418	167040220	168787679	825702227	**Plasma**		0.710	100	3.524	100	96.48
Pre-topup-3	2077417	169785427	168787679	825702227	**Buffer**	0.025					

Post-topup-1	63830789	174349633	168787679	825702227	**Plasma**		0.748	100	2.877	100	97.12
Post-topup-1	1874084	177909307	168787679	825702227	**Buffer**	0.022					
Post-topup-2	73627877	172416499	168787679	825702227	**Plasma**		0.873	100	3.476	100	96.52
Post-topup-2	2561734	172567563	168787679	825702227	**Buffer**	0.030					
Post-topup-3	64481147	170767143	168787679	825702227	**Plasma**		0.772	100	3.044	100	96.96
Post-topup-3	1879923	163555696	168787679	825702227	**Buffer**	0.023					
Pre-Rescue-1	58760963	171897125	168787679	825702227	**Plasma**		0.699	100	5.371	100	94.63
Pre-Rescue-1	3141421	171094283	168787679	825702227	**Buffer**	0.038					
Post-Rescue-1	64394687	174263473	168787679	825702227	**Plasma**		0.755	100	5.380	100	94.62
Post-Rescue-1	3311873	166587177	168787679	825702227	**Buffer**	0.041					

Patient-3											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	308920	139794238	1023214069	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	136180633	139794238	1023214069	**Buffer**	0.000					
5 mins	103326900	107526171	139794238	1023214069	**Plasma**		1.313	100	2.298	100	97.70
5mins	3312563	149980794	139794238	1023214069	**Buffer**	0.030					
10 mins	106042303	113609897	139794238	1023214069	**Plasma**		1.275	100	2.408	100	97.59
10 mins	2834622	126132783	139794238	1023214069	**Buffer**	0.031					
15 mins	124269371	118387439	139794238	1023214069	**Plasma**		1.434	100	0.252	100	99.75
15 mins	3863848	1462101125	139794238	1023214069	**Buffer**	0.004					
20 mins	120506411	121148527	139794238	1023214069	**Plasma**		1.359	100	2.087	100	97.91
20 mins	3123614	150498975	139794238	1023214069	**Buffer**	0.028					
25 mins	115124334	114834194	139794238	1023214069	**Plasma**		1.370	100	2.457	100	97.54
25 mins	3603133	146270863	139794238	1023214069	**Buffer**	0.034					
30 mins	109170212	117935895	139794238	1023214069	**Plasma**		1.265	100	2.275	100	97.73
30 mins	3131845	148740230	139794238	1023214069	**Buffer**	0.029					
1 hrs	101222208	111159153	139794238	1023214069	**Plasma**		1.244	100	1.741	100	98.26
1 hrs	2322679	146513180	139794238	1023214069	**Buffer**	0.022					
4 hrs	95032387	110884756	139794238	1023214069	**Plasma**		1.171	100	2.679	100	97.32

4 hrs	3343826	145612617	139794238	1023214069	**Buffer**	0.031					
24 hrs	142967558	108326159	139794238	1023214069	**Plasma**		1.803	100	1.666	100	98.33
24 hrs	3156635	143529441	139794238	1023214069	**Buffer**	0.030					
Pre-topup-1	99865809	112510975	139794238	1023214069	**Plasma**		1.213	100	2.360	100	97.64
Pre-topup-1	3077771	146901783	139794238	1023214069	**Buffer**	0.029					
Pre-topup-2	119417167	107216000	139794238	1023214069	**Plasma**		1.522	100	2.020	100	97.98
Pre-topup-2	3339297	148451298	139794238	1023214069	**Buffer**	0.031					
Pre-topup-3	115265474	106672866	139794238	1023214069	**Plasma**		1.476	100	1.649	100	98.35
Pre-topup-3	2623927	147272743	139794238	1023214069	**Buffer**	0.024					
Post-topup-1	121160761	101995677	139794238	1023214069	**Plasma**		1.623	100	1.653	100	98.35
Post-topup-1	2839604	144574792	139794238	1023214069	**Buffer**	0.027					
Post-topup-2	125463614	90961882	139794238	1023214069	**Plasma**		1.884	100	1.657	100	98.34
Post-topup-2	3363256	147128065	139794238	1023214069	**Buffer**	0.031					

Post-topup-3	143445607	116253990	139794238	1023214069	**Plasma**		1.686	100	1.241	100	98.76
Post-topup-3	2225351	145318465	139794238	1023214069	**Buffer**	0.021					

Patient-4K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
						µg/ml	µg/ml				
Baseline	0	152268531	127760794	959779222	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	145519307	127760794	959779222	**Buffer**	0.000					
5 mins	129228639	146459011	127760794	959779222	**Plasma**		1.175	100	2.380	100	97.62
5mins	3103735	147779867	127760794	959779222	**Buffer**	0.028					
10 mins	153095450	141503513	127760794	959779222	**Plasma**		1.440	100	2.117	100	97.88
10 mins	3400568	148493792	127760794	959779222	**Buffer**	0.030					
15 mins	170771867	139352402	127760794	959779222	**Plasma**		1.631	100	2.338	100	97.66
15 mins	4309160	150414409	127760794	959779222	**Buffer**	0.038					
20 mins	157592565	136649273	127760794	959779222	**Plasma**		1.535	100	2.516	100	97.48
20 mins	4332252	149322882	127760794	959779222	**Buffer**	0.039					
25 mins	154764646	142537741	127760794	959779222	**Plasma**		1.445	100	2.612	100	97.39
25 mins	4337414	152966423	127760794	959779222	**Buffer**	0.038					

30 mins	147749069	132644430	127760794	959779222	**Plasma**		1.483	100	2.196	100	97.80
30 mins	3632015	148451319	127760794	959779222	**Buffer**	0.033					
1 hrs	143294748	140258113	127760794	959779222	**Plasma**		1.360	100	2.150	100	97.85
1 hrs	3288140	149672520	127760794	959779222	**Buffer**	0.029					
24 hrs	68894533	130049161	127760794	959779222	**Plasma**		0.705	100	0.755	100	99.24
24 hrs	569602	142355293	127760794	959779222	**Buffer**	0.005					
Pre-topup-1	91643607	137830643	127760794	959779222	**Plasma**		0.885	100	2.181	100	97.82
Pre-topup-1	2170240	149686218	127760794	959779222	**Buffer**	0.019					
Pre-topup-2	102983814	148865446	127760794	959779222	**Plasma**		0.921	100	1.699	100	98.30
Pre-topup-2	1679181	142847885	127760794	959779222	**Buffer**	0.016					
Pre-topup-3	76888292	143104152	127760794	959779222	**Plasma**		0.715	100	1.376	100	98.62
Pre-topup-3	1066795	144338032	127760794	959779222	**Buffer**	0.010					
Post-topup-1	113823071	145299716	127760794	959779222	**Plasma**		1.043	100	1.801	100	98.20

Post-topup-1	2106257	149293634	127760794	959779222	**Buffer**	0.019					
Post-topup-2	89722933	148809927	127760794	959779222	**Plasma**		0.803	100	1.907	100	98.09
Post-topup-2	1693522	147252213	127760794	959779222	**Buffer**	0.015					
Post-topup-3	106786350	141295302	127760794	959779222	**Plasma**		1.006	100	0.516	100	99.48
Post-topup-3	581774	149154112	127760794	959779222	**Buffer**	0.005					

Patient-5K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	102340499	127760794	959779222	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	94977637	127760794	959779222	**Buffer**	0.000					
5 mins	44052164	91167437	127760794	959779222	**Plasma**		0.643	100	2.166	100	97.83
5mins	978639	93493553	127760794	959779222	**Buffer**	0.014					
10 mins	54859694	90622229	127760794	959779222	**Plasma**		0.806	100	2.079	100	97.92
10 mins	1163953	92470886	127760794	959779222	**Buffer**	0.017					
15 mins	67460108	89277353	127760794	959779222	**Plasma**		1.006	100	2.203	100	97.808
15 mins	1577660	94761232	127760794	959779222	**Buffer**	0.022					

20 mins	72800471	87196060	127760794	959779222	**Plasma**		1.111	100	2.513	100	97.49
20 mins	1971306	93954256	127760794	959779222	**Buffer**	0.028					
25 mins	76577830	85921473	127760794	959779222	**Plasma**		1.186	100	2.777	100	97.22
25 mins	2260623	91326563	127760794	959779222	**Buffer**	0.033					
30 mins	83433704	86211388	127760794	959779222	**Plasma**		1.288	100	2.803	100	97.208
30 mins	2455015	90499693	127760794	959779222	**Buffer**	0.036					
1 hrs	90993386	87755155	127760794	959779222	**Plasma**		1.380	100	2.298	100	97.70
1 hrs	2217803	93083152	127760794	959779222	**Buffer**	0.032					
24 hrs	101001011	83058527	127760794	959779222	**Plasma**		1.619	100	1.413	100	98.59
24 hrs	1558483	90719045	127760794	959779222	**Buffer**	0.023					
Pre-topup-2	87312305	83346220	127760794	959779222	**Plasma**		1.394	100	2.407	100	97.59
Pre-topup-2	2370628	94033695	127760794	959779222	**Buffer**	0.034					
Pre-topup-3	67102691	86101735	127760794	959779222	**Plasma**		1.037	100	1.629	100	98.37
Pre-topup-3	1151683	90688786	127760794	959779222	**Buffer**	0.017					
Post-topup-1	83072803	87244019	127760794	959779222	**Plasma**		1.268	100	2.275	100	97.72
Post-topup-1	2006647	92633382	127760794	959779222	**Buffer**	0.029					

Post-topup-3	95702345	81108406	127760794	959779222	**Plasma**		1.571	100	1.348	100	98.65
Post-topup-3	1458602	91686195	127760794	959779222	**Buffer**	0.021					

Patient-6k											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	135931378	127760794	959779222	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	103536770	127760794	959779222	**Buffer**	0.000					
5 mins	56493717	90573756	127760794	959779222	**Plasma**		0.830	100	3.786	100	96.21
5mins	2265569	95939081	127760794	959779222	**Buffer**	0.031					
10 mins	70442405	89667669	127760794	959779222	**Plasma**		1.046	100	4.216	100	95.78
10 mins	3028252	91427391	127760794	959779222	**Buffer**	0.044					
15 mins	68813426	92439031	127760794	959779222	**Plasma**		0.991	100	3.962	100	96.04
15 mins	2629500	89151623	127760794	959779222	**Buffer**	0.039					
20 mins	65318954	89809859	127760794	959779222	**Plasma**		0.968	100	3.871	100	96.13
20 mins	2559005	90898859	127760794	959779222	**Buffer**	0.037					
25 mins	63003067	90548516	127760794	959779222	**Plasma**		0.926	100	3.886	100	96.11
25 mins	2468771	91310812	127760794	959779222	**Buffer**	0.036					
30 mins	66200720	85804527	127760794	959779222	**Plasma**		1.027	100	4.201	100	95.80

30 mins	3006041	92741671	127760794	959779222	**Buffer**	0.043					
1 hrs	67538915	96621666	127760794	959779222	**Plasma**		0.930	100	4.166	100	95.83
1 hrs	2781451	95513113	127760794	959779222	**Buffer**	0.039					
4 hrs	75672070	84032914	127760794	959779222	**Plasma**		1.199	100	2.609	100	97.39
4 hrs	2104891	89585673	127760794	959779222	**Buffer**	0.031					
24 hrs	112984049	83096803	127760794	959779222	**Plasma**		1.810	100	2.568	100	97.43
24 hrs	2976698	85266247	127760794	959779222	**Buffer**	0.046					
Pre-topup-1	87140427	89203672	127760794	959779222	**Plasma**		1.300	100	2.860	100	97.14
Pre-topup-1	2491994	89201518	127760794	959779222	**Buffer**	0.037					
Pre-topup-2	89169816	86674107	127760794	959779222	**Plasma**		1.369	100	1.973	100	98.03
Pre-topup-2	1859420	91583752	127760794	959779222	**Buffer**	0.027					
Pre-topup-3	88901087	82180898	127760794	959779222	**Plasma**		1.440	100	2.523	100	97.48
Pre-topup-3	2419083	88616296	127760794	959779222	**Buffer**	0.036					
Post-topup-1	103395377	85501134	127760794	959779222	**Plasma**		1.610	100	2.698	100	97.30
Post-topup-1	2897889	88804961	127760794	959779222	**Buffer**	0.043					

Post-topup-2	94241821	83055749	127760794	959779222	**Plasma**		1.510	100	3.590	100	96.41
Post-topup-2	3665603	89998822	127760794	959779222	**Buffer**	0.054					
Post-topup-3	112633852	84428415	127760794	959779222	**Plasma**		1.776	100	2.814	100	97.19
Post-topup-3	3311607	88222910	127760794	959779222	**Buffer**	0.050					

Patient-7k											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
						µg/ml	µg/ml				
Baseline	0	391212650	111729233	111601420	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	175072471	111729233	111601420	**Buffer**	0.000					
5 mins	100634431	137212633	111729233	111601420	**Plasma**		0.734	100	2.169	100	97.83
5mins	1827021	114848903	111729233	111601420	**Buffer**	0.016					
10 mins	128641277	140873468	111729233	111601420	**Plasma**		0.914	100	2.942	100	97.06
10 mins	3067281	114175433	111729233	111601420	**Buffer**	0.027					
15 mins	146475286	127183631	111729233	111601420	**Plasma**		1.153	100	2.170	100	97.83
15 mins	2811256	112476982	111729233	111601420	**Buffer**	0.025					
20 mins	153563033	138154606	111729233	111601420	**Plasma**		1.113	100	2.424	100	97.58
20 mins	3039857	112845487	111729233	111601420	**Buffer**	0.027					

25 mins	165928039	134829702	111729233	111601420	**Plasma**		1.232	100	2.498	100	97.50
25 mins	3455387	112393424	111729233	111601420	**Buffer**	0.031					
30 mins	162640180	144065623	111729233	111601420	**Plasma**		1.130	100	2.281	100	97.72
30 mins	2913875	113143602	111729233	111601420	**Buffer**	0.026					
1 hrs	144969204	125136713	111729233	111601420	**Plasma**		1.160	100	2.178	100	97.82
1 hrs	2795274	110759360	111729233	111601420	**Buffer**	0.025					
4 hrs	91800547	104504129	111729233	111601420	**Plasma**		0.879	100	1.237	100	98.76
4 hrs	1178780	108472530	111729233	111601420	**Buffer**	0.011					
24 hrs	114085956	103171340	111729233	111601420	**Plasma**		1.107	100	0.613	100	99.39
24 hrs	733799	108233210	111729233	111601420	**Buffer**	0.007					
Pre-topup-1	86443746	110512891	111729233	111601420	**Plasma**		0.783	100	1.171	100	98.83
Pre-topup-1	986866	107765014	111729233	111601420	**Buffer**	0.009					
Pre-topup-2	75104964	104949290	111729233	111601420	**Plasma**		0.716	100	1.204	100	98.80
Pre-topup-2	916088	106335488	111729233	111601420	**Buffer**	0.009					
Pre-topup-3	67765032	109888382	111729233	111601420	**Plasma**		0.617	100	0.307	100	99.69
Pre-topup-3	199686	105328041	111729233	111601420	**Buffer**	0.002					

Post-topup-1	94034324	106798392	111729233	111601420	**Plasma**		0.881	100	1.365	100	98.63
Post-topup-1	1294091	107683695	111729233	111601420	**Buffer**	0.012					
Post-topup-3	138589283	101539353	111729233	111601420	**Plasma**		1.366	100	0.757	100	99.24
Post-topup-3	1102472	106651119	111729233	111601420	**Buffer**	0.010					

Patient-8k											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	106528648	111729233	111601420	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	113523895	111729233	111601420	**Buffer**	0.000					
5 mins	195065643	108949718	111729233	111601420	**Plasma**		1.792	100	2.793	100	97.21
5mins	5353458	107041793	111729233	111601420	**Buffer**	0.050					
10 mins	191434743	107968995	111729233	111601420	**Plasma**		1.775	100	3.173	100	96.83
10 mins	6097020	108366006	111729233	111601420	**Buffer**	0.056					
15 mins	237941816	108496091	111729233	111601420	**Plasma**		2.196	100	2.717	100	97.28
15 mins	6359090	106701912	111729233	111601420	**Buffer**	0.060					
20 mins	241343214	104805058	111729233	111601420	**Plasma**		2.305	100	3.116	100	96.88

20 mins	7549785	105221971	111729233	111601420	**Buffer**	0.072					
25 mins	230916350	107018316	111729233	111601420	**Plasma**		2.160	100	2.895	100	97.10
25 mins	6521324	104403940	111729233	111601420	**Buffer**	0.063					
30 mins	212927252	99764534	111729233	111601420	**Plasma**		2.137	100	2.680	100	97.32
30 mins	6106340	106746053	111729233	111601420	**Buffer**	0.057					
1 hrs	196732026	101689497	111729233	111601420	**Plasma**		1.937	100	2.303	100	97.70
1 hrs	4690876	105271811	111729233	111601420	**Buffer**	0.045					
24 hrs	33725882	98534881	111729233	111601420	**Plasma**		0.343	100	0.616	100	99.38
24 hrs	215804	102355378	111729233	111601420	**Buffer**	0.002					
Pre-topup-1	123567974	102096359	111729233	111601420	**Plasma**		1.212	100	1.779	100	98.22
Pre-topup-1	2174594	101014662	111729233	111601420	**Buffer**	0.022					
Pre-topup-2	95898277	101820793	111729233	111601420	**Plasma**		0.943	100	1.898	100	98.10
Pre-topup-2	1823976	102038752	111729233	111601420	**Buffer**	0.018					
Pre-topup-3	63584689	103656133	111729233	111601420	**Plasma**		0.614	100	1.000	100	99.00
Pre-topup-3	642255	104361464	111729233	111601420	**Buffer**	0.006					

Post-topup-1	142516586	100475838	111729233	111601420	**Plasma**		1.420	100	1.889	100	98.11
Post-topup-1	2795526	104361464	111729233	111601420	**Buffer**	0.027					
Post-topup-2	86826420	101413147	111729233	111601420	**Plasma**		0.857	100	1.900	100	98.10
Post-topup-2	1703751	104494038	111729233	111601420	**Buffer**	0.016					
Post-topup-3	55511296	102965617	111729233	111601420	**Plasma**		0.540	100	0.448	100	99.55
Post-topup-3	249567	103387291	111729233	111601420	**Buffer**	0.002					

Patient-9k											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
						µg/ml	µg/ml				
Baseline	0	695155589	111729233	111601420	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	423174944	111729233	111601420	**Buffer**	0.000					
5 mins	122937195	133072303	111729233	111601420	**Plasma**		0.925	100	3.057	100	96.94
5mins	2970949	105186524	111729233	111601420	**Buffer**	0.028					
10 mins	133133827	123060800	111729233	111601420	**Plasma**		1.083	100	2.872	100	97.13
10 mins	3092573	99520249	111729233	111601420	**Buffer**	0.031					
15 mins	131684129	116584833	111729233	111601420	**Plasma**		1.131	100	2.870	100	97.13

15 mins	3159051	97458117	111729233	111601420	**Buffer**	0.032					
20 mins	132732871	117398355	111729233	111601420	**Plasma**		1.132	100	2.687	100	97.31
20 mins	2731979	89936751	111729233	111601420	**Buffer**	0.030					
25 mins	129959092	113029809	111729233	111601420	**Plasma**		1.151	100	2.812	100	97.19
25 mins	3059988	94637551	111729233	111601420	**Buffer**	0.032					
30 mins	129661574	111935275	111729233	111601420	**Plasma**		1.160	100	2.984	100	97.02
30 mins	3351656	96963082	111729233	111601420	**Buffer**	0.035					
			111729233	111601420							
1 hrs	112566651	111339334	111729233	111601420	**Plasma**		1.012	100	2.325	100	97.68
1 hrs	2342902	99684765	111729233	111601420	**Buffer**	0.024					
24 hrs	141821776	99689386	111729233	111601420	**Plasma**		1.424	100	1.549	100	98.45
24 hrs	2292491	104019575	111729233	111601420	**Buffer**	0.022					
Pre-topup-1	94119233	108196980	111729233	111601420	**Plasma**		0.871	100	1.973	100	98.01
Pre-topup-1	1615530	94113344	111729233	111601420	**Buffer**	0.017					
Pre-topup-2	106774561	110104865	111729233	111601420	**Plasma**		0.971	100	1.601	100	98.40
Pre-topup-2	1514700	97588115	111729233	111601420	**Buffer**	0.016					
Pre-topup-3	112471819	106082318	111729233	111601420	**Plasma**		1.061	100	2.669	100	97.33
Pre-	2633763	93065799	111729233	111601420	**Buffer**	0.028					

topup-3											
Post-topup-1	117936217	102268791	111729233	111601420	**Plasma**		1.155	100	1.773	100	98.23
Post-topup-1	1923199	94046862	111729233	111601420	**Buffer**	0.020					
Post-topup-2	96002647	105630615	111729233	111601420	**Plasma**		0.910	100	2.577	100	97.42
Post-topup-2	2233639	95379671	111729233	111601420	**Buffer**	0.023					
Post-topup-3	146797857	102235898	111729233	111601420	**Plasma**		1.438	100	2.262	100	97.74
Post-topup-3	3463771	106667234	111729233	111601420	**Buffer**	0.033					

Patient-10k											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	167197030	111729233	111601420	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	105877355	111729233	111601420	**Buffer**	0.000					
5 mins	74904686	326335027	111729233	111601420	**Plasma**		0.230	100	5.423	100	94.58
5mins	1812769	145619845	111729233	111601420	**Buffer**	0.012					
10 mins	107845503	117719597	111729233	111601420	**Plasma**		0.917	100	2.495	100	97.50
10 mins	2320009	101501339	111729233	111601420	**Buffer**	0.023					

15 mins	134659557	112415396	111729233	111601420	**Plasma**		1.199	100	2.343	100	97.66
15 mins	2843659	101301530	111729233	111601420	**Buffer**	0.028					
20 mins	132450017	107974559	111729233	111601420	**Plasma**		1.228	100	2.244	100	97.76
20 mins	2691251	97760609	111729233	111601420	**Buffer**	0.028					
25 mins	133476675	107126614	111729233	111601420	**Plasma**		1.247	100	1.913	100	98.09
25 mins	2461547	103250425	111729233	111601420	**Buffer**	0.024					
30 mins	139370611	107008295	111729233	111601420	**Plasma**		1.304	100	1.900	100	98.10
30 mins	2471470	99877452	111729233	111601420	**Buffer**	0.025					
			111729233	111601420							
1 hrs	134641562	108820928	111729233	111601420	**Plasma**		1.239	100	2.091	100	97.91
1 hrs	2549143	98532281	111729233	111601420	**Buffer**	0.026					
24 hrs	163167657	97256636	111729233	111601420	**Plasma**		1.680	100	1.410	100	98.59
24 hrs	2383682	100766143	111729233	111601420	**Buffer**	0.024					
Pre-topup-1	128006686	117705927	111729233	111601420	**Plasma**		1.089	100	2.359	100	97.64
Pre-topup-1	2584869	100773603	111729233	111601420	**Buffer**	0.026					
Pre-topup-2	106774561	110104865	111729233	111601420	**Plasma**		0.971	100	1.601	100	98.40
Pre-topup-2	1514700	97588115	111729233	111601420	**Buffer**	0.016					
Pre-	112231543	129166612	111729233	111601420	**Plasma**		0.870	100	1.698	100	98.30

topup-3											
Pre-topup-3	1582132	107253966	111729233	111601420	**Buffer**	0.015					
Post-topup-1	161122509	103998343	111729233	111601420	**Plasma**		1.551	100	2.019	100	97.98
Post-topup-1	3128543	100024768	111729233	111601420	**Buffer**	0.031					
Post-topup-2	96002647	105630615	111729233	111601420	**Plasma**		0.910	100	2.577	100	97.42
Post-topup-2	2233639	95379671	111729233	111601420	**Buffer**	0.023					
Post-topup-3	159951018	110305096	111729233	111601420	**Plasma**		1.452	100	1.660	100	98.34
Post-topup-3	2455027	102008670	111729233	111601420	**Buffer**	0.024					

Patient-11K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	398645978	115697530	107332655	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	132706023	115697530	107332655	**Buffer**	0.000					

5 mins	93227405	121787693	115697530	107332655	**Plasma**		0.825	100	1.566	100	98.43
5mins	1261159	105184129	115697530	107332655	**Buffer**	0.013					
10 mins	100205453	128994390	115697530	107332655	**Plasma**		0.837	100	1.785	100	98.21
10 mins	1506012	108602517	115697530	107332655	**Buffer**	0.015					
15 mins	114075691	128065977	115697530	107332655	**Plasma**		0.960	100	1.436	100	98.56
15 mins	1373009	107334512	115697530	107332655	**Buffer**	0.014					
20 mins	121998757	127285833	115697530	107332655	**Plasma**		1.033	100	1.373	100	98.63
20 mins	1438582	109301355	115697530	107332655	**Buffer**	0.014					
25 mins	125911487	125255393	115697530	107332655	**Plasma**		1.084	100	1.444	100	98.56
25 mins	1609775	110897839	115697530	107332655	**Buffer**	0.016					
30 mins	134467532	125424775	115697530	107332655	**Plasma**		1.156	100	1.394	100	98.61
30 mins	1650343	110404647	115697530	107332655	**Buffer**	0.016					
1 hrs	130343644	123465237	115697530	107332655	**Plasma**		1.138	100	1.243	100	98.76
1 hrs	1419477	108130152	115697530	107332655	**Buffer**	0.014					

24 hrs	168187864	114096646	115697530	107332655	**Plasma**		1.589	100	0.651	100	99.35
24 hrs	1105271	115233488	115697530	107332655	**Buffer**	0.010					
Pre-topup-1	151552293	122054404	115697530	107332655	**Plasma**		1.338	100	1.319	100	98.68
Pre-topup-1	1833869	111956749	115697530	107332655	**Buffer**	0.018					
Pre-topup-2	220761189	113745491	115697530	107332655	**Plasma**		2.092	100	0.906	100	99.09
Pre-topup-2	1901288	108153021	115697530	107332655	**Buffer**	0.019					
Pre-topup-3	129996821	114761620	115697530	107332655	**Plasma**		1.221	100	0.736	100	99.26
Pre-topup-3	900371	108031733	115697530	107332655	**Buffer**	0.009					
Post-topup-1	169326989	119026059	115697530	107332655	**Plasma**		1.533	100	1.108	100	98.89
Post-topup-1	1819900	115408632	115697530	107332655	**Buffer**	0.017					
Post-topup-2	250639672	115605822	115697530	107332655	**Plasma**		2.337	100	1.487	100	98.51
Post-topup-2	3583191	111179409	115697530	107332655	**Buffer**	0.035					

Post-topup-3	164445585	115467284	115697530	107332655	**Plasma**		1.535	100	0.660	100	99.34
Post-topup-3	1039758	110613983	115697530	107332655	**Buffer**	0.010					

Patient-12K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
						µg/ml	µg/ml				
Baseline	0	132913505	112581616	91462277	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	100907052	112581616	91462277	**Buffer**	0.000					
5 mins	32335683	297740066	112581616	91462277	**Plasma**		0.134	100	5.785	100	94.21
5mins	910459	144921776	112581616	91462277	**Buffer**	0.008					
10 mins	56507637	156138023	112581616	91462277	**Plasma**		0.445	100	3.515	100	96.49
10 mins	1316580	103507568	112581616	91462277	**Buffer**	0.016					
15 mins	85014500	130709004	112581616	91462277	**Plasma**		0.801	100	3.735	100	96.27
15 mins	2508028	103253167	112581616	91462277	**Buffer**	0.030					

20 mins	91242547	122098187	112581616	91462277	**Plasma**		0.920	100	3.572	100	96.43
20 mins	2676093	100261368	112581616	91462277	**Buffer**	0.033					
25 mins	98746299	123944557	112581616	91462277	**Plasma**		0.981	100	3.520	100	96.48
25 mins	2725694	97206186	112581616	91462277	**Buffer**	0.035					
30 mins	94981806	130368733	112581616	91462277	**Plasma**		0.897	100	3.194	100	96.81
30 mins	2325933	99944429	112581616	91462277	**Buffer**	0.029					
1 hrs	125815413	113063790	112581616	91462277	**Plasma**		1.370	100	2.919	100	97.08
1 hrs	3144283	96807430	112581616	91462277	**Buffer**	0.040					
24 hrs	95332187	103424969	112581616	91462277	**Plasma**		1.135	100	1.865	100	98.13
24 hrs	1612527	93789170	112581616	91462277	**Buffer**	0.021					
Pre-topup-1	113863596	119627490	112581616	91462277	**Plasma**		1.172	100	2.683	100	97.32
Pre-topup-1	2538705	99406084	112581616	91462277	**Buffer**	0.031					
Post-topup-1	137715208	107984997	112581616	91462277	**Plasma**		1.570	100	2.336	100	97.66

Post-topup-1	2876097	96545480	112581616	91462277	**Buffer**	0.037					

Patient-13k											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
Baseline	0	149898610	112581616	91462277	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	114559312	129561457	101738555	**Buffer**	0.000					
5 mins	27735807	143308123	129561457	101738555	**Plasma**		0.246	100	6.536	100	93.46
5mins	1521375	120277157	129561457	101738555	**Buffer**	0.016					
10 mins	53003158	150424482	129561457	101738555	**Plasma**		0.449	100	3.500	100	96.50
10 mins	1484817	120394076	129561457	101738555	**Buffer**	0.016					
15 mins	62681451	150744279	129561457	101738555	**Plasma**		0.530	100	3.460	100	96.54
15 mins	1763656	122597682	129561457	101738555	**Buffer**	0.018					
20 mins	67649245	152561565	129561457	101738555	**Plasma**		0.565	100	3.649	100	96.35
20 mins	2002660	123770639	129561457	101738555	**Buffer**	0.021					
25 mins	66379524	145512255	129561457	101738555	**Plasma**		0.581	100	2.950	100	97.05

25 mins	1670471	124148999	129561457	101738555	**Buffer**	0.017					
30 mins	80309130	142697165	129561457	101738555	**Plasma**		0.717	100	2.863	100	97.14
30 mins	1948073	120898118	129561457	101738555	**Buffer**	0.021					
1 hrs	122947345	136279069	129561457	101738555	**Plasma**		1.149	100	2.622	100	97.38
1 hrs	2834339	119814480	129561457	101738555	**Buffer**	0.030					
24 hrs	187504348	134245673	129561457	101738555	**Plasma**		1.779	100	2.120	100	97.88
24 hrs	3541451	119625228	129561457	101738555	**Buffer**	0.038					
Pre-topup-1	176046010	134490863	129561457	101738555	**Plasma**		1.667	100	2.649	100	97.35
Pre-topup-1	4252312	122655982	129561457	101738555	**Buffer**	0.044					
Pre-topup-2	211314435	132315796	129561457	101738555	**Plasma**		2.034	100	2.362	100	97.64
Pre-topup-2	4740524	125695154	129561457	101738555	**Buffer**	0.048					
Post-topup-1	197400367	131661585	129561457	101738555	**Plasma**		1.909	100	2.195	100	97.80
Post-topup-1	4212365	127987276	129561457	101738555	**Buffer**	0.042					

Post-topup-2	216525331	130564853	129561457	101738555	**Plasma**		2.112	100	1.926	100	98.07
Post-topup-2	3984217	124755799	129561457	101738555	**Buffer**	0.041					

Patient-14K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
						µg/ml	µg/ml				
Baseline	0	148578600	111384600	114692280	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	120261193	111384600	114692280	**Buffer**	0.000					
5 mins	68879371	133239538	111384600	114692280	**Plasma**		0.502	100	2.693	100	97.31
5mins	1724417	123845832	111384600	114692280	**Buffer**	0.014					
10 mins	105419663	128644468	111384600	114692280	**Plasma**		0.796	100	3.002	100	97.00
10 mins	2780693	113024979	111384600	114692280	**Buffer**	0.024					
15 mins	122518935	126234266	111384600	114692280	**Plasma**		0.943	100	3.096	100	96.90
15 mins	3627797	120714488	111384600	114692280	**Buffer**	0.029					
20 mins	136834893	120731872	111384600	114692280	**Plasma**		1.101	100	2.991	100	97.01

20 mins	4176493	123218134	111384600	114692280	**Buffer**	0.033					
25 mins	114996392	108459359	111384600	114692280	**Plasma**		1.030	100	3.330	100	96.67
25 mins	4340561	122939733	111384600	114692280	**Buffer**	0.034					
30 mins	124390863	115025918	111384600	114692280	**Plasma**		1.050	100	3.433	100	96.57
30 mins	4629307	124679652	111384600	114692280	**Buffer**	0.036					
1 hrs	126448214	115101677	111384600	114692280	**Plasma**		1.067	100	3.082	100	96.92
1 hrs	3956585	116853363	111384600	114692280	**Buffer**	0.033					
24 hrs	145425532	109157199	111384600	114692280	**Plasma**		1.294	100	1.994	100	98.01
24 hrs	3118375	117366905	111384600	114692280	**Buffer**	0.026					
Pre-topup-1	134892906	111888865	111384600	114692280	**Plasma**		1.171	100	2.730	100	97.27
Pre-topup-1	3945582	119874273	111384600	114692280	**Buffer**	0.032					
Pre-topup-2	159355881	114459599	111384600	114692280	**Plasma**		1.352	100	2.373	100	97.63
Pre-topup-2	3854463	116643817	111384600	114692280	**Buffer**	0.032					

Pre-topup-3	135362476	111445164	111384600	114692280	**Plasma**		1.180	100	2.984	100	97.02
Pre-topup-3	4223197	116534707	111384600	114692280	**Buffer**	0.035					
Post-topup-1	135170253	116458995	111384600	114692280	**Plasma**		1.127	100	2.739	100	97.26
Post-topup-1	3675786	115604788	111384600	114692280	**Buffer**	0.031					
Post-topup-2	135410851	121599412	111384600	114692280	**Plasma**		1.081	100	3.007	100	96.99
Post-topup-2	3866751	115489490	111384600	114692280	**Buffer**	0.033					
Post-topup-3	137431036	121509364	111384600	114692280	**Plasma**		1.098	100	3.207	100	96.79
Post-topup-3	4045588	111551385	111384600	114692280	**Buffer**	0.035					

Patient-15K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
						µg/ml	µg/ml				
Baseline	0	111929800	126111662	120478808	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	104481473	126111662	120478808	**Buffer**	0.000					

5 mins	54035609	116106065	126111662	120478808	**Plasma**		0.487	100	2.063	100	97.94
5mins	1005560	104728642	126111662	120478808	**Buffer**	0.010					
10 mins	59680149	119060516	126111662	120478808	**Plasma**		0.525	100	2.128	100	97.87
10 mins	1084775	101702411	126111662	120478808	**Buffer**	0.011					
15 mins	57625182	114832692	126111662	120478808	**Plasma**		0.525	100	2.273	100	97.73
15 mins	1107679	97108361	126111662	120478808	**Buffer**	0.012					
20 mins	69403509	113183887	126111662	120478808	**Plasma**		0.642	100	2.036	100	97.96
20 mins	1324381	106074731	126111662	120478808	**Buffer**	0.013					
25 mins	74412272	109567538	126111662	120478808	**Plasma**		0.711	100	2.044	100	97.96
25 mins	1438221	103611526	126111662	120478808	**Buffer**	0.015					
30 mins	99556936	113680990	126111662	120478808	**Plasma**		0.917	100	2.533	100	97.47
30 mins	2329812	105039540	126111662	120478808	**Buffer**	0.023					
1 hrs	118420563	107059505	126111662	120478808	**Plasma**		1.158	100	2.791	100	97.21
1 hrs	3017703	97752540	126111662	120478808	**Buffer**	0.032					

24 hrs	104797066	103729195	126111662	120478808	**Plasma**		1.058	100	1.490	100	98.51
24 hrs	1493183	99162943	126111662	120478808	**Buffer**	0.016					
Pre-topup-1	129094968	105601985	126111662	120478808	**Plasma**		1.280	100	2.322	100	97.68
Pre-topup-1	2967171	104527630	126111662	120478808	**Buffer**	0.030					
Pre-topup-2	155285806	107127521	126111662	120478808	**Plasma**		1.517	100	2.148	100	97.85
Pre-topup-2	3356006	107802440	126111662	120478808	**Buffer**	0.033					
Pre-topup-3	125018507	110677698	126111662	120478808	**Plasma**		1.182	100	1.701	100	98.30
Pre-topup-3	1993479	103733192	126111662	120478808	**Buffer**	0.020					
Post-topup-1	166004754	103004976	126111662	120478808	**Plasma**		1.687	100	2.274	100	97.73
Post-topup-1	3982144	108653265	126111662	120478808	**Buffer**	0.038					
Post-topup-2	161113626	104039077	126111662	120478808	**Plasma**		1.621	100	1.850	100	98.15
Post-topup-2	3096957	108077783	126111662	120478808	**Buffer**	0.030					

Post-topup-3	115550061	109692903	126111662	120478808	**Plasma**		1.103	100	1.598	100	98.40
Post-topup-3	1885209	112004975	126111662	120478808	**Buffer**	0.018					

Patient-16K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	93891140	101200324	93062747	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	86544824	101200324	93062747	**Buffer**	0.000					
5 mins	84442234	93971953	101200324	93062747	**Plasma**		0.977	100	2.307	100	97.69
5mins	1889089	91142237	101200324	93062747	**Buffer**	0.023					
10 mins	133703950	95387613	101200324	93062747	**Plasma**		1.524	100	4.246	100	95.75
10 mins	3989730	67034615	101200324	93062747	**Buffer**	0.065					
15 mins	153680434	94024753	101200324	93062747	**Plasma**		1.777	100	2.742	100	97.26
15 mins	4152393	92641667	101200324	93062747	**Buffer**	0.049					
20 mins	159349461	89464759	101200324	93062747	**Plasma**		1.937	100	3.019	100	96.98

20 mins	4785640	88984857	101200324	93062747	**Buffer**	0.058					
25 mins	160087038	90605666	101200324	93062747	**Plasma**		1.921	100	3.009	100	96.99
25 mins	4899793	92150513	101200324	93062747	**Buffer**	0.058					
30 mins	160323566	92303747	101200324	93062747	**Plasma**		1.889	100	3.253	100	96.75
30 mins	5176981	91615735	101200324	93062747	**Buffer**	0.061					
1 hrs	174302180	93389131	101200324	93062747	**Plasma**		2.030	100	3.175	100	96.82
1 hrs	5234651	88341908	101200324	93062747	**Buffer**	0.064					
24 hrs	47421345	89781364	101200324	93062747	**Plasma**		0.574	100	0.714	100	99.29
24 hrs	336978	89348657	101200324	93062747	**Buffer**	0.004					
Pre-topup-1	123741043	88040418	101200324	93062747	**Plasma**		1.528	100	2.055	100	97.94
Pre-topup-1	2720177	94164283	101200324	93062747	**Buffer**	0.031					
Pre-topup-2	119763369	90631122	101200324	93062747	**Plasma**		1.437	100	1.901	100	98.10
Pre-topup-2	2276960	90628613	101200324	93062747	**Buffer**	0.027					

Pre-topup-3	67264296	90574063	101200324	93062747	**Plasma**		0.808	100	2.552	100	97.45
Pre-topup-3	1022981	53980544	101200324	93062747	**Buffer**	0.021					
Post-topup-1	126591692	97457819	101200324	93062747	**Plasma**		1.413	100	2.192	100	97.81
Post-topup-1	2541641	89280816	101200324	93062747	**Buffer**	0.031					
Post-topup-2	135484312	89504550	101200324	93062747	**Plasma**		1.646	100	1.553	100	98.45
Post-topup-2	2185640	92962213	101200324	93062747	**Buffer**	0.026					
Post-topup-3	55187764	93345149	101200324	93062747	**Plasma**		0.129	100	1.497	100	98.50
Post-topup-3	803197	90780482	101200324	93062747	**Buffer**	0.002					

Patient-17K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	153207864	94426566	89643886	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	97914968	94426566	89643886	**Buffer**	0.000					

5 mins	88309045	98211434	94426566	89643886	**Plasma**		0.947	100	3.483	100	96.52
5mins	2780112	88772389	94426566	89643886	**Buffer**	0.033					
10 mins	117729333	91952816	94426566	89643886	**Plasma**		1.349	100	3.664	100	96.33
10 mins	4055175	86434753	94426566	89643886	**Buffer**	0.049					
15 mins	116287037	56841851	94426566	89643886	**Plasma**		2.155	100	2.148	100	97.85
15 mins	3895954	88656544	94426566	89643886	**Buffer**	0.046					
20 mins	126020479	104464191	94426566	89643886	**Plasma**		1.271	100	3.520	100	96.48
20 mins	3626789	85420006	94426566	89643886	**Buffer**	0.045					
25 mins	124678522	101028714	94426566	89643886	**Plasma**		1.300	100	3.660	100	96.34
25 mins	3916461	86700776	94426566	89643886	**Buffer**	0.048					
30 mins	120614875	97138795	94426566	89643886	**Plasma**		1.308	100	3.667	100	96.33
30 mins	4091148	89847127	94426566	89643886	**Buffer**	0.048					
1 hrs	118094378	91506034	94426566	89643886	**Plasma**		1.359	100	3.018	100	96.98
1 hrs	3521342	90412929	94426566	89643886	**Buffer**	0.041					

24 hrs	121226704	60021062	94426566	89643886	**Plasma**		2.127	100	1.694	100	98.31
24 hrs	2152515	62912619	94426566	89643886	**Buffer**	0.036					
Pre-topup-1	153938005	89253259	94426566	89643886	**Plasma**		1.817	100	2.254	100	97.75
Pre-topup-1	3396008	87351813	94426566	89643886	**Buffer**	0.041					
Pre-topup-2	162162195	88078641	94426566	89643886	**Plasma**		1.939	100	2.581	100	97.42
Pre-topup-2	4072780	85699629	94426566	89643886	**Buffer**	0.050					
Pre-topup-3	168762333	87696923	94426566	89643886	**Plasma**		2.027	100	1.507	100	98.49
Pre-topup-3	2412640	83175360	94426566	89643886	**Buffer**	0.031					
Post-topup-1	183650778	92018812	94426566	89643886	**Plasma**		2.102	100	3.546	100	96.45
Post-topup-1	4284653	60535066	94426566	89643886	**Buffer**	0.075					
Post-topup-2	168973294	88737050	94426566	89643886	**Plasma**		2.006	100	2.671	100	97.33
Post-topup-2	4300928	84554971	94426566	89643886	**Buffer**	0.054					

Post-topup-3	171797021	88422168	94426566	89643886	**Plasma**		0.409	100	1.836	100	98.16
Post-topup-3	3112526	87238762	94426566	89643886	**Buffer**	0.008					

Patient-18K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	63278211	68930924	53099225	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	70087413	68930924	53099225	**Buffer**	0.000					
5 mins	79867762	75435145	68930924	53099225	**Plasma**		1.374	100	0.308	100	99.69
5mins	234939	72109146	68930924	53099225	**Buffer**	0.004					
10 mins	119486428	73154078	68930924	53099225	**Plasma**		2.120	100	0.471	100	99.53
10 mins	562374	73068287	68930924	53099225	**Buffer**	0.010					
15 mins	120256372	104656846	68930924	53099225	**Plasma**		1.492	100	0.884	100	99.12
15 mins	719414	70863718	68930924	53099225	**Buffer**	0.013					
20 mins	129793221	79049428	68930924	53099225	**Plasma**		2.131	100	0.581	100	99.42

20 mins	739809	77560533	68930924	53099225	**Buffer**	0.012					
25 mins	131986308	79208114	68930924	53099225	**Plasma**		2.163	100	0.417	100	99.58
25 mins	263660	37966633	68930924	53099225	**Buffer**	0.009					
30 mins	144794013	78411952	68930924	53099225	**Plasma**		2.397	100	1.275	100	98.72
30 mins	1009805	42883597	68930924	53099225	**Buffer**	0.031					
1 hrs	165026345	104886279	68930924	53099225	**Plasma**		2.042	100	0.541	100	99.46
1 hrs	591839	69523531	68930924	53099225	**Buffer**	0.011					
			68930924	53099225							
24 hrs	91466676	72744989	68930924	53099225	**Plasma**		1.632	100	0.147	100	99.85
24 hrs	131418	71111532	68930924	53099225	**Buffer**	0.002					
			68930924	53099225							
Pre-topup-1	168793975	76008442	68930924	53099225	**Plasma**		2.883	100	0.468	100	99.53
Pre-topup-1	771833	74309505	68930924	53099225	**Buffer**	0.013					
			68930924	53099225							
Pre-topup-2	165480695	67508371	68930924	53099225	**Plasma**		3.182	100	0.642	100	99.36
Pre-topup-2	1118245	71009146	68930924	53099225	**Buffer**	0.020					
			68930924	53099225							

Pre-topup-3	80159428	75997773	68930924	53099225	**Plasma**		1.369	100	0.321	100	99.68
Pre-topup-3	255478	75394190	68930924	53099225	**Buffer**	0.004					
			68930924	53099225							
Post-topup-1	200680193	65126596	68930924	53099225	**Plasma**		4.000	100	0.451	100	99.55
Post-topup-1	1087640	78259431	68930924	53099225	**Buffer**	0.018					
			68930924	53099225							
Post-topup-2	153087074	74577713	68930924	53099225	**Plasma**		2.665	100	0.716	100	99.28
Post-topup-2	1047856	71305777	68930924	53099225	**Buffer**	0.019					
			68930924	53099225							
Post-topup-3	119473991	70343978	68930924	53099225	**Plasma**		0.441	100	0.227	100	99.77
Post-topup-3	290433	75296827	68930924	53099225	**Buffer**	0.001					

Patient-19K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	524236517	89792797	100982825	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	227537265	89792797	100982825	**Buffer**	0.000					

5 mins	28929485	105478784	89792797	100982825	**Plasma**		0.244	100	1.124	100	98.88
5mins	294805	95607368	89792797	100982825	**Buffer**	0.003					
10 mins	54183184	103545464	89792797	100982825	**Plasma**		0.465	100	2.948	100	97.05
10 mins	1395154	90436536	89792797	100982825	**Buffer**	0.014					
15 mins	58904808	111608224	89792797	100982825	**Plasma**		0.469	100	2.917	100	97.08
15 mins	1379075	89586653	89792797	100982825	**Buffer**	0.014					
20 mins	77714875	102830757	89792797	100982825	**Plasma**		0.672	100	2.721	100	97.28
20 mins	1792221	87149723	89792797	100982825	**Buffer**	0.018					
25 mins	92550229	103095745	89792797	100982825	**Plasma**		0.798	100	3.067	100	96.93
25 mins	2324630	84444351	89792797	100982825	**Buffer**	0.024					
25 mins-2	92784979	103314055	89792797	100982825	**Plasma**		0.799	100	2.878	100	97.12
25 mins-2	2212449	85612796	89792797	100982825	**Buffer**	0.023					
25 mins-3	92954285	106782045	89792797	100982825	**Plasma**		0.774	100	2.338	100	97.66
25 mins-3	1748319	85917579	89792797	100982825	**Buffer**	0.018					

25 mins-4	73386861	108405136	89792797	100982825	**Plasma**		0.602	100	3.634	100	96.37
25 mins-4	2270866	92317775	89792797	100982825	**Buffer**	0.022					
25 mins-5	92247714	108440313	89792797	100982825	**Plasma**		0.756	100	2.901	100	97.10
25 mins-5	2207043	89431127	89792797	100982825	**Buffer**	0.022					
30 mins	93488941	105514518	89792797	100982825	**Plasma**		0.788	100	2.976	100	97.02
30 mins	2279075	86440634	89792797	100982825	**Buffer**	0.023					
1 hrs	132764592	112899066	89792797	100982825	**Plasma**		1.046	100	2.888	100	97.11
1 hrs	3017574	88840695	89792797	100982825	**Buffer**	0.030					
24 hrs	146184794	89429770	89792797	100982825	**Plasma**		1.453	100	2.199	100	97.80
24 hrs	3005857	83614458	89792797	100982825	**Buffer**	0.032					
Pre-topup-1	138820475	96501838	89792797	100982825	**Plasma**		1.279	100	2.766	100	97.23
Pre-topup-1	3376823	84872688	89792797	100982825	**Buffer**	0.035					
Pre-topup-3	127211479	89794143	89792797	100982825	**Plasma**		1.260	100	1.924	100	98.08

Pre-topup-3	2305285	84591701	89792797	100982825	**Buffer**	0.024					
Post-topup-1	151409817	91034971	89792797	100982825	**Plasma**		1.479	100	2.949	100	97.05
Post-topup-1	4344742	88578520	89792797	100982825	**Buffer**	0.044					
Post-topup-3	138212781	86107793	89792797	100982825	**Plasma**		0.285	100	2.425	100	97.58
Post-topup-3	3392579	87162768	89792797	100982825	**Buffer**	0.007					

Patient-20K											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	105468451	105152672	102482853	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	96194893	105152672	102482853	**Buffer**	0.000					
5 mins	63658000	113250473	105152672	102482853	**Plasma**		0.577	100	2.708	100	97.29
5mins	1569648	103101296	105152672	102482853	**Buffer**	0.016					

10 mins	82266337	106865053	105152672	102482853	**Plasma**		0.790	100	2.872	100	97.13
10 mins	2346940	106140436	105152672	102482853	**Buffer**	0.023					
15 mins	118727685	119759098	105152672	102482853	**Plasma**		1.017	100	3.296	100	96.70
15 mins	2836784	86823232	105152672	102482853	**Buffer**	0.034					
20 mins	146724880	131218162	105152672	102482853	**Plasma**		1.147	100	3.210	100	96.79
20 mins	3269762	91095633	105152672	102482853	**Buffer**	0.037					
25 mins	151729681	129914696	105152672	102482853	**Plasma**		1.198	100	3.221	100	96.78
25 mins	3340614	88802426	105152672	102482853	**Buffer**	0.039					
30 mins	159486072	124631341	105152672	102482853	**Plasma**		1.313	100	2.934	100	97.07
30 mins	3276112	87264865	105152672	102482853	**Buffer**	0.039					
1 hrs	164223054	123328870	105152672	102482853	**Plasma**		1.366	100	2.730	100	97.27
1 hrs	3238873	89093019	105152672	102482853	**Buffer**	0.037					
24 hrs	146184794	89429770	105152672	102482853	**Plasma**		1.677	100	2.199	100	97.80
24 hrs	3005857	83614458	105152672	102482853	**Buffer**	0.037					
Pre-topup-1	164215609	113347414	105152672	102482853	**Plasma**		1.487	100	1.922	100	98.08

Pre-topup-1	2482188	89161474	105152672	102482853	**Buffer**	0.029					
Pre-topup-2	207249787	109452437	105152672	102482853	**Plasma**		1.943	100	2.568	100	97.43
Pre-topup-2	4111256	84556084	105152672	102482853	**Buffer**	0.050					
Pre-topup-3	174903416	107865165	105152672	102482853	**Plasma**		1.664	100	2.258	100	97.74
Pre-topup-3	3030764	82784092	105152672	102482853	**Buffer**	0.038					
Post-topup-1	184665187	112578708	105152672	102482853	**Plasma**		1.683	100	2.644	100	97.36
Post-topup-1	3856709	88913151	105152672	102482853	**Buffer**	0.045					
Post-topup-3	223878157	106770773	105152672	102482853	**Plasma**		0.430	100	2.381	100	97.62
Post-topup-3	4350118	87124140	105152672	102482853	**Buffer**	0.010					

Knee joint surgery patients
Batch 2

Patient-1											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**%Bound**
						µg/ml	µg/ml				
Baseline	0	76572402	68040216	68440438	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	70581547	68040216	68440438	**Buffer**	0.000					
5 mins	62470230	79675321	68040216	68440438	**Plasma**		0.779	100	3.725	100	96.28
5mins	2050605	70202061	68040216	68440438	**Buffer**	0.029					
10 mins	1.43E+08	77696433	68040216	68440438	**Plasma**		1.827	100	5.681	100	94.32
10 mins	7275244	69675059	68040216	68440438	**Buffer**	0.104					
15 mins	1.11E+08	80977913	68040216	68440438	**Plasma**		1.368	100	4.991	100	95.01
15 mins	4971357	72409215	68040216	68440438	**Buffer**	0.068					
20 mins	1.27E+08	78152585	68040216	68440438	**Plasma**		1.622	100	5.620	100	94.38
20 mins	6529247	71218109	68040216	68440438	**Buffer**	0.091					
25 mins	1.29E+08	79906235	68040216	68440438	**Plasma**		1.605	100	5.249	100	94.75
25 mins	6011780	70946506	68040216	68440438	**Buffer**	0.084					
30 mins	1.34E+08	79317251	68040216	68440438	**Plasma**		1.682	100	4.336	100	95.66

30 mins	5225748	71229587	68040216	68440438	**Buffer**	0.073					
1 hrs	1.18E+08	77729930	68040216	68440438	**Plasma**		1.509	100	4.706	100	95.29
1 hrs	5142863	72009936	68040216	68440438	**Buffer**	0.071					
4 hrs	98146895	76930372	68040216	68440438	**Plasma**		1.268	100	3.794	100	96.21
4 hrs	3410106	70444853	68040216	68440438	**Buffer**	0.048					
12 hrs	78518889	76644579	68040216	68440438	**Plasma**		1.018	100	3.915	100	96.09
12 hrs	2854862	71175976	68040216	68440438	**Buffer**	0.040					
24 hrs	1.24E+08	73971292	68040216	68440438	**Plasma**		1.665	100	3.289	100	96.71
24 hrs	3856276	70026127	68040216	68440438	**Buffer**	0.055					

Patient-2											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	210206889	68040216	68440438	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	87213144	68040216	68440438	**Buffer**	0.000					
5 mins	72755615	103288515	68040216	68440438	**Plasma**		0.700	100	4.172	100	95.83
5mins	2233825	76007737	68040216	68440438	**Buffer**	0.029					
10 mins	101241452	94029461	68040216	68440438	**Plasma**		1.070	100	4.216	100	95.78
10 mins	3370574	74248925	68040216	68440438	**Buffer**	0.045					

15 mins	131758077	91169571	68040216	68440438	**Plasma**		1.437	100	4.498	100	95.50
15 mins	4758431	73207075	68040216	68440438	**Buffer**	0.065					
20 mins	136514111	94527856	68040216	68440438	**Plasma**		1.436	100	4.841	100	95.16
20 mins	4902214	70124653	68040216	68440438	**Buffer**	0.069					
25 mins	132943443	92875930	68040216	68440438	**Plasma**		1.423	100	4.812	100	95.19
25 mins	4889508	70992089	68040216	68440438	**Buffer**	0.068					
30 mins	132617232	93469484	68040216	68440438	**Plasma**		1.411	100	4.725	100	95.28
30 mins	4989317	74418149	68040216	68440438	**Buffer**	0.067					
1 hrs	147389881	89693183	68040216	68440438	**Plasma**		1.634	100	3.880	100	96.12
1 hrs	4743116	74396067	68040216	68440438	**Buffer**	0.063					
4 hrs	111864059	76420696	68040216	68440438	**Plasma**		1.455	100	2.819	100	97.18
4 hrs	2956059	71636860	68040216	68440438	**Buffer**	0.041					
12 hrs	105528322	75554967	68040216	68440438	**Plasma**		1.389	100	2.603	100	97.40
12 hrs	2662817	73241558	68040216	68440438	**Buffer**	0.036					
24 hrs	116228776	73960381	68040216	68440438	**Plasma**		1.562	100	1.779	100	98.22
24 hrs	1990648	71200080	68040216	68440438	**Buffer**	0.028					

Patient-3											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	65394499	50504766	53037014	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	51175492	50504766	53037014	**Buffer**	0.000					
5 mins	10254403	69199517	50504766	53037014	**Plasma**		0.141	100	4.366	100	95.63
5mins	327826	50666118	50504766	53037014	**Buffer**	0.006					
10 mins	22920851	70305970	50504766	53037014	**Plasma**		0.310	100	5.017	100	94.98
10 mins	855595	52311627	50504766	53037014	**Buffer**	0.016					
15 mins	28778504	68202415	50504766	53037014	**Plasma**		0.402	100	4.884	100	95.12
15 mins	1191738	57824562	50504766	53037014	**Buffer**	0.020					
20 mins	29207971	69093634	50504766	53037014	**Plasma**		0.403	100	4.820	100	95.18
20 mins	1166981	57278795	50504766	53037014	**Buffer**	0.019					
25 mins	32113817	68535904	50504766	53037014	**Plasma**		0.446	100	4.923	100	95.08
25 mins	1337008	57955897	50504766	53037014	**Buffer**	0.022					
30 mins	33726969	68209634	50504766	53037014	**Plasma**		0.471	100	4.930	100	95.07
30 mins	1382962	56727221	50504766	53037014	**Buffer**	0.023					
1 hrs	39175492	66752470	50504766	53037014	**Plasma**		0.559	100	4.826	100	95.17
1 hrs	1581761	55850287	50504766	53037014	**Buffer**	0.027					
4 hrs	57754904	65151483	50504766	53037014	**Plasma**		0.844	100	4.735	100	95.27

4 hrs	2492824	59387108	50504766	53037014	**Buffer**	0.040					
12 hrs	81165363	61783694	50504766	53037014	**Plasma**		1.251	100	5.921	100	94.08
12 hrs	4528997	58222705	50504766	53037014	**Buffer**	0.074					
24 hrs	1.12E+08	58332079	50504766	53037014	**Plasma**		1.824	100	3.715	100	96.29
24 hrs	4116244	57827349	50504766	53037014	**Buffer**	0.068					

Patient-4											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	90506944	68040216	68440438	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	86498561	68040216	68440438	**Buffer**	0.000					
5 mins	66777103	95940545	68040216	68440438	**Plasma**		0.692	100	4.099	100	95.90
5mins	2412825	84578759	68040216	68440438	**Buffer**	0.028					
10 mins	1.14E+08	95251823	68040216	68440438	**Plasma**		1.187	100	5.679	100	94.32
10 mins	5244703	77334134	68040216	68440438	**Buffer**	0.067					
15 mins	1.35E+08	88867963	68040216	68440438	**Plasma**		1.515	100	5.843	100	94.16
15 mins	7927754	89010980	68040216	68440438	**Buffer**	0.089					
20 mins	1.61E+08	95526699	68040216	68440438	**Plasma**		1.681	100	5.155	100	94.84
20 mins	7750044	88925778	68040216	68440438	**Buffer**	0.087					

25 mins	1.42E+08	97475222	68040216	68440438	**Plasma**		1.445	100	5.833	100	94.17
25 mins	7487088	88331742	68040216	68440438	**Buffer**	0.084					
30 mins	1.29E+08	1.01E+08	68040216	68440438	**Plasma**		1.272	100	5.274	100	94.73
30 mins	6041722	89546639	68040216	68440438	**Buffer**	0.067					
1 hrs	1.42E+08	95242509	68040216	68440438	**Plasma**		1.484	100	4.447	100	95.55
1 hrs	5810227	87524486	68040216	68440438	**Buffer**	0.066					
4 hrs	94889973	98105880	68040216	68440438	**Plasma**		0.962	100	3.755	100	96.24
4 hrs	3154125	86841829	68040216	68440438	**Buffer**	0.036					
12 hrs	87036505	92142055	68040216	68440438	**Plasma**		0.939	100	2.832	100	97.17
12 hrs	2328996	87049119	68040216	68440438	**Buffer**	0.027					
24 hrs	74879242	90226504	68040216	68440438	**Plasma**		0.825	100	0.899	100	99.10
24 hrs	617747	82821346	68040216	68440438	**Buffer**	0.007					

Patient-5											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	96895582	69482705	63515378	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	70519974	69482705	63515378	**Buffer**	0.000					
5 mins	46420186	89720702	69482705	63515378	**Plasma**		0.566	100	2.530	100	97.47

5mins	953811	72868736	69482705	63515378	**Buffer**	0.014					
10 mins	54084888	88637853	69482705	63515378	**Plasma**		0.668	100	2.322	100	97.68
10 mins	1063364	75049807	69482705	63515378	**Buffer**	0.015					
15 mins	51518595	89018067	69482705	63515378	**Plasma**		0.633	100	2.204	100	97.80
15 mins	926023	72592600	69482705	63515378	**Buffer**	0.014					
20 mins	46946237	95623445	69482705	63515378	**Plasma**		0.537	100	2.424	100	97.58
20 mins	904632	76014987	69482705	63515378	**Buffer**	0.013					
25 mins	43299290	93161278	69482705	63515378	**Plasma**		0.508	100	2.256	100	97.74
25 mins	795293	75853346	69482705	63515378	**Buffer**	0.011					
30 mins	41233034	88729764	69482705	63515378	**Plasma**		0.508	100	1.767	100	98.23
30 mins	635515	77403685	69482705	63515378	**Buffer**	0.009					
1 hrs	26878212	87177549	69482705	63515378	**Plasma**		0.337	100	2.494	100	97.51
1 hrs	615493	80031100	69482705	63515378	**Buffer**	0.008					
4 hrs	42423915	82001513	69482705	63515378	**Plasma**		0.566	100	2.021	100	97.98
4 hrs	823503	78766105	69482705	63515378	**Buffer**	0.011					
12 hrs	56506481	77027073	69482705	63515378	**Plasma**		0.803	100	1.761	100	98.24
12 hrs	972418	75264845	69482705	63515378	**Buffer**	0.014					
24 hrs	1.12E+08	77217708	69482705	63515378	**Plasma**		1.586	100	1.721	100	98.28
24 hrs	1913290	76691360	69482705	63515378	**Buffer**	0.027					

Patient-6											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	144751562	65293090	64485006	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	78630927	65293090	64485006	**Buffer**	0.000					
5 mins	18639027	76199830	65293090	64485006	**Plasma**		0.248	100	3.118	100	96.88
5mins	505614	66303387	65293090	64485006	**Buffer**	0.008					
10 mins	34725807	76675179	65293090	64485006	**Plasma**		0.459	100	4.285	100	95.71
10 mins	1285399	66230646	65293090	64485006	**Buffer**	0.020					
15 mins	36917641	75846195	65293090	64485006	**Plasma**		0.493	100	4.254	100	95.75
15 mins	1371490	66236722	65293090	64485006	**Buffer**	0.021					
20 mins	36820531	76708658	65293090	64485006	**Plasma**		0.486	100	4.203	100	95.80
20 mins	1387143	68758061	65293090	64485006	**Buffer**	0.020					
25 mins	36375832	75351920	65293090	64485006	**Plasma**		0.489	100	4.077	100	95.92
25 mins	1360371	69113952	65293090	64485006	**Buffer**	0.020					
30 mins	35184369	77108562	65293090	64485006	**Plasma**		0.462	100	4.001	100	96.00
30 mins	1251573	68558232	65293090	64485006	**Buffer**	0.018					

1 hrs	35539920	76897742	65293090	64485006	**Plasma**		0.468	100	3.413	100	96.59
1 hrs	1057881	67061239	65293090	64485006	**Buffer**	0.016					
4 hrs	44752803	70366027	65293090	64485006	**Plasma**		0.644	100	3.941	100	96.06
4 hrs	1601221	63886561	65293090	64485006	**Buffer**	0.025					
12 hrs	66874939	109534685	65293090	64485006	**Plasma**		0.618	100	6.130	100	93.87
12 hrs	2795456	74698619	65293090	64485006	**Buffer**	0.038					
24 hrs	124422077	65198617	65293090	64485006	**Plasma**		1.932	100	4.149	100	95.85
24 hrs	4966794	62734001	65293090	64485006	**Buffer**	0.080					

Patient-7											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	109726166	67225921	64906484	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	74521254	67225921	64906484	**Buffer**	0.000					
5 mins	45907667	84339474	67225921	64906484	**Plasma**		0.564	100	6.270	100	93.73
5mins	2495708	73123054	67225921	64906484	**Buffer**	0.035					
10 mins	56384063	86982550	67225921	64906484	**Plasma**		0.671	100	7.277	100	92.72
10 mins	3589914	76102881	67225921	64906484	**Buffer**	0.049					
15 mins	57108305	81800898	67225921	64906484	**Plasma**		0.723	100	6.460	100	93.54

15 mins	3408972	75585979	67225921	64906484	**Buffer**	0.047					
20 mins	56123134	85108197	67225921	64906484	**Plasma**		0.683	100	6.028	100	93.97
20 mins	3044556	76594403	67225921	64906484	**Buffer**	0.041					
25 mins	50534227	85240269	67225921	64906484	**Plasma**		0.614	100	6.251	100	93.75
25 mins	2882457	77778533	67225921	64906484	**Buffer**	0.038					
30 mins	49116824	88634695	67225921	64906484	**Plasma**		0.574	100	6.123	100	93.88
30 mins	2675367	78854633	67225921	64906484	**Buffer**	0.035					
1 hrs	51062306	87367068	67225921	64906484	**Plasma**		0.605	100	5.715	100	94.29
1 hrs	2544623	76176120	67225921	64906484	**Buffer**	0.035					
4 hrs	56682850	75225455	67225921	64906484	**Plasma**		0.780	100	5.398	100	94.60
4 hrs	3078913	75695406	67225921	64906484	**Buffer**	0.042					

Patient-8											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	60675668	88613163	78878115	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	81109699	88613163	78878115	**Buffer**	0.000					
5 mins	83136809	72077092	88613163	78878115	**Plasma**		1.296	100	5.697	100	94.30
5mins	4639089	70596266	88613163	78878115	**Buffer**	0.074					

10 mins	95963710	74442223	88613163	78878115	**Plasma**		1.448	100	6.177	100	93.82
10 mins	5943998	74648947	88613163	78878115	**Buffer**	0.089					
15 mins	93058160	72860402	88613163	78878115	**Plasma**		1.435	100	6.093	100	93.91
15 mins	5400907	69398519	88613163	78878115	**Buffer**	0.087					
20 mins	87147276	68370955	88613163	78878115	**Plasma**		1.432	100	5.434	100	94.57
20 mins	4813598	69492584	88613163	78878115	**Buffer**	0.078					
25 mins	78294338	74155932	88613163	78878115	**Plasma**		1.186	100	4.907	100	95.09
25 mins	3617490	69817258	88613163	78878115	**Buffer**	0.058					
30 mins	76978117	73108122	88613163	78878115	**Plasma**		1.183	100	5.283	100	94.72
30 mins	3893587	69993971	88613163	78878115	**Buffer**	0.062					
1 hrs	68130231	73557821	88613163	78878115	**Plasma**		1.041	100	4.504	100	95.50
1 hrs	2919134	69977105	88613163	78878115	**Buffer**	0.047					
4 hrs	55933003	69168002	88613163	78878115	**Plasma**		0.908	100	3.722	100	96.28
4 hrs	2104630	69930429	88613163	78878115	**Buffer**	0.034					
12 hrs	61968821	70883340	88613163	78878115	**Plasma**		0.982	100	3.282	100	96.72
12 hrs	1948428	67904596	88613163	78878115	**Buffer**	0.032					
24 hrs	73348423	69547736	88613163	78878115	**Plasma**		1.185	100	2.889	100	97.11
24 hrs	2080788	68290257	88613163	78878115	**Buffer**	0.034					

Patient-9											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	1.39E+08	86633473	64583295	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	97200666	86633473	64583295	**Buffer**	0.000					
5 mins	26678718	93907308	86633473	64583295	**Plasma**		0.381	100	7.178	100	92.82
5mins	1743315	85492441	86633473	64583295	**Buffer**	0.027					
10 mins	31834462	1.01E+08	86633473	64583295	**Plasma**		0.423	100	7.811	100	92.19
10 mins	2217775	90034852	86633473	64583295	**Buffer**	0.033					
15 mins	51186492	1.01E+08	86633473	64583295	**Plasma**		0.682	100	7.381	100	92.62
15 mins	3373266	89945951	86633473	64583295	**Buffer**	0.050					
20 mins	49476388	99336584	86633473	64583295	**Plasma**		0.668	100	6.976	100	93.02
20 mins	3131919	90137888	86633473	64583295	**Buffer**	0.047					
25 mins	59462044	99847059	86633473	64583295	**Plasma**		0.799	100	7.711	100	92.29
25 mins	4143476	90231169	86633473	64583295	**Buffer**	0.062					
30 mins	62240073	1.01E+08	86633473	64583295	**Plasma**		0.827	100	6.889	100	93.11
30 mins	3696416	87006666	86633473	64583295	**Buffer**	0.057					

1 hrs	84449215	1.04E+08	86633473	64583295	**Plasma**		1.091	100	7.191	100	92.81
1 hrs	5165882	88311870	86633473	64583295	**Buffer**	0.078					
4 hrs	94848275	96209423	86633473	64583295	**Plasma**		1.322	100	5.547	100	94.45
4 hrs	4623937	84556670	86633473	64583295	**Buffer**	0.073					
12 hrs	96437800	95833114	86633473	64583295	**Plasma**		1.350	100	5.418	100	94.58
12 hrs	4753398	87189599	86633473	64583295	**Buffer**	0.073					
24 hrs	1.04E+08	91120325	86633473	64583295	**Plasma**		1.529	100	4.622	100	95.38
24 hrs	4498513	85372959	86633473	64583295	**Buffer**	0.071					

Patient-10											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	102706666	88613163	78878115	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	85832800	88613163	78878115	**Buffer**	0.000					
5 mins	70839662	99646241	88613163	78878115	**Plasma**		0.799	100	3.483	100	96.52
5mins	2322827	93810692	88613163	78878115	**Buffer**	0.028					
10 mins	101991085	99204718	88613163	78878115	**Plasma**		1.155	100	3.680	100	96.32
10 mins	3352152	88604482	88613163	78878115	**Buffer**	0.043					
15 mins	102870794	99944183	88613163	78878115	**Plasma**		1.156	100	4.130	100	95.87

15 mins	3721009	87528479	88613163	78878115	**Buffer**	0.048					
20 mins	100428293	100685006	88613163	78878115	**Plasma**		1.121	100	3.923	100	96.08
20 mins	4109742	105031680	88613163	78878115	**Buffer**	0.044					
25 mins	89571940	101922139	88613163	78878115	**Plasma**		0.987	100	4.360	100	95.64
25 mins	3331736	86959948	88613163	78878115	**Buffer**	0.043					
30 mins	92285386	102880795	88613163	78878115	**Plasma**		1.008	100	4.329	100	95.67
30 mins	3452857	88926987	88613163	78878115	**Buffer**	0.044					
1 hrs	137830884	97944052	88613163	78878115	**Plasma**		1.581	100	3.190	100	96.81
1 hrs	3881088	86464655	88613163	78878115	**Buffer**	0.050					
4 hrs	140789521	91476308	88613163	78878115	**Plasma**		1.729	100	3.153	100	96.85
4 hrs	4361571	89874092	88613163	78878115	**Buffer**	0.055					
12 hrs	169841790	92996043	88613163	78878115	**Plasma**		2.052	100	3.887	100	96.11
12 hrs	6196852	87301718	88613163	78878115	**Buffer**	0.080					
24 hrs	159233643	90703184	88613163	78878115	**Plasma**		1.972	100	2.324	100	97.68
24 hrs	3587488	87939534	88613163	78878115	**Buffer**	0.046					

Patient-12											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	103187576	86879450	72381995	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	81692451	86879450	72381995	**Buffer**	0.000					
5 mins	39296509	97642764	86879450	72381995	**Plasma**		0.483	100	1.972	100	98.03
5mins	677172	85312602	86879450	72381995	**Buffer**	0.010					
10 mins	67664311	96702487	86879450	72381995	**Plasma**		0.840	100	2.211	100	97.79
10 mins	1384938	89503988	86879450	72381995	**Buffer**	0.019					
15 mins	76324722	90632402	86879450	72381995	**Plasma**		1.011	100	2.110	100	97.89
15 mins	1538215	86579155	86879450	72381995	**Buffer**	0.021					
20 mins	77948300	94956495	86879450	72381995	**Plasma**		0.985	100	1.925	100	98.08
20 mins	1395078	88276447	86879450	72381995	**Buffer**	0.019					
25 mins	78744696	95244127	86879450	72381995	**Plasma**		0.992	100	1.910	100	98.09
25 mins	1388345	87917809	86879450	72381995	**Buffer**	0.019					
30 mins	75522410	93922256	86879450	72381995	**Plasma**		0.965	100	1.913	100	98.09
30 mins	1308379	85075983	86879450	72381995	**Buffer**	0.018					
1 hrs	66281157	95072748	86879450	72381995	**Plasma**		0.837	100	1.758	100	98.24

1 hrs	1024426	83564713	86879450	72381995	**Buffer**	0.015					
4 hrs	79547667	90654095	86879450	72381995	**Plasma**		1.053	100	1.491	100	98.51
4 hrs	1116387	85311294	86879450	72381995	**Buffer**	0.016					
12 hrs	122432621	87369087	86879450	72381995	**Plasma**		1.682	100	1.351	100	98.65
12 hrs	1712325	90448784	86879450	72381995	**Buffer**	0.023					
24 hrs	159686002	92422228	86879450	72381995	**Plasma**		2.074	100	1.686	100	98.31
24 hrs	2507652	86095190	86879450	72381995	**Buffer**	0.035					

Patient-13											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	150474872	99833246	78954793	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	107641931	99833246	78954793	**Buffer**	0.000					
5 mins	87942238	118498507	99833246	78954793	**Plasma**		0.938	100	2.777	100	97.22
5mins	2125043	103123131	99833246	78954793	**Buffer**	0.026					
10 mins	109866905	133458925	99833246	78954793	**Plasma**		1.041	100	3.151	100	96.85
10 mins	2722705	104967186	99833246	78954793	**Buffer**	0.033					
15 mins	73988497	133256347	99833246	78954793	**Plasma**		0.702	100	4.791	100	95.21
15 mins	2892983	108745274	99833246	78954793	**Buffer**	0.034					

20 mins	134654034	129894076	99833246	78954793	**Plasma**		1.311	100	3.228	100	96.77
20 mins	3546428	105997054	99833246	78954793	**Buffer**	0.042					
25 mins	96284231	143206929	99833246	78954793	**Plasma**		0.850	100	3.456	100	96.54
25 mins	2595746	111710544	99833246	78954793	**Buffer**	0.029					
30 mins	132318439	131161792	99833246	78954793	**Plasma**		1.276	100	3.344	100	96.66
30 mins	3675542	108954910	99833246	78954793	**Buffer**	0.043					
1 hrs	133485811	124514923	99833246	78954793	**Plasma**		1.356	100	2.644	100	97.36
1 hrs	2952722	104161159	99833246	78954793	**Buffer**	0.036					
4 hrs	159313139	115907310	99833246	78954793	**Plasma**		1.738	100	2.791	100	97.21
4 hrs	3927376	102386308	99833246	78954793	**Buffer**	0.049					
12 hrs	137555289	106994024	99833246	78954793	**Plasma**		1.626	100	2.351	100	97.65
12 hrs	3055396	101081767	99833246	78954793	**Buffer**	0.038					
24 hrs	139964231	102730699	99833246	78954793	**Plasma**		1.723	100	1.430	100	98.57
24 hrs	1955412	100335577	99833246	78954793	**Buffer**	0.025					

Patient-14											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	109917391	91188740	72551325	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	93131456	91188740	72551325	**Buffer**	0.000					
5 mins	58055416	96698703	91188740	72551325	**Plasma**		0.755	100	2.235	100	97.77
5mins	1243597	92676959	91188740	72551325	**Buffer**	0.017					
10 mins	73105622	94190271	91188740	72551325	**Plasma**		0.976	100	2.552	100	97.45
10 mins	1752248	88473842	91188740	72551325	**Buffer**	0.025					
15 mins	87907261	95294887	91188740	72551325	**Plasma**		1.159	100	2.658	100	97.34
15 mins	2196237	89568156	91188740	72551325	**Buffer**	0.031					
20 mins	88672871	96314354	91188740	72551325	**Plasma**		1.157	100	2.768	100	97.23
20 mins	2302837	90377780	91188740	72551325	**Buffer**	0.032					
25 mins	91961478	94425064	91188740	72551325	**Plasma**		1.224	100	3.005	100	97.00
25 mins	2579541	88131907	91188740	72551325	**Buffer**	0.037					
30 mins	92115411	98932221	91188740	72551325	**Plasma**		1.170	100	3.007	100	96.99
30 mins	2557804	91348846	91188740	72551325	**Buffer**	0.035					
1 hrs	95738477	98719096	91188740	72551325	**Plasma**		1.219	100	3.326	100	96.67

1 hrs	2680508	83107081	91188740	72551325	**Buffer**	0.041					
4 hrs	90619544	93086413	91188740	72551325	**Plasma**		1.224	100	2.424	100	97.58
4 hrs	1977965	83810194	91188740	72551325	**Buffer**	0.030					
12 hrs	125848587	94890025	91188740	72551325	**Plasma**		1.667	100	3.985	100	96.01
12 hrs	4355472	82404798	91188740	72551325	**Buffer**	0.066					
24 hrs	120256601	90958157	91188740	72551325	**Plasma**		1.662	100	2.060	100	97.94
24 hrs	2294161	84249739	91188740	72551325	**Buffer**	0.034					

Patient-15											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	79874968	60250379	54354820	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	58130107	60250379	54354820	**Buffer**	0.000					
5 mins	39285912	66585798	60250379	54354820	**Plasma**		0.654	100	5.258	100	94.74
5mins	1825707	58848361	60250379	54354820	**Buffer**	0.034					
10 mins	53008962	68337888	60250379	54354820	**Plasma**		0.860	100	5.598	100	94.40
10 mins	2628745	60542416	60250379	54354820	**Buffer**	0.048					
15 mins	55877713	67820897	60250379	54354820	**Plasma**		0.913	100	5.764	100	94.24
15 mins	2903902	61148331	60250379	54354820	**Buffer**	0.053					

20 mins	54533192	67814636	60250379	54354820	**Plasma**		0.891	100	5.223	100	94.78
20 mins	2564138	61047843	60250379	54354820	**Buffer**	0.047					
25 mins	52192115	70561317	60250379	54354820	**Plasma**		0.820	100	4.906	100	95.09
25 mins	2269213	62538501	60250379	54354820	**Buffer**	0.040					
30 mins	52907123	66684762	60250379	54354820	**Plasma**		0.879	100	4.612	100	95.39
30 mins	2228862	60918118	60250379	54354820	**Buffer**	0.041					
1 hrs	53568611	68178441	60250379	54354820	**Plasma**		0.871	100	3.948	100	96.05
1 hrs	1902689	61336815	60250379	54354820	**Buffer**	0.034					
4 hrs	62427336	67471182	60250379	54354820	**Plasma**		1.026	100	3.437	100	96.56
4 hrs	1983929	62382100	60250379	54354820	**Buffer**	0.035					
12 hrs	81492993	65191369	60250379	54354820	**Plasma**		1.386	100	2.577	100	97.42
12 hrs	1991983	61836619	60250379	54354820	**Buffer**	0.036					
24 hrs	82373457	62851663	60250379	54354820	**Plasma**		1.453	100	2.718	100	97.28
24 hrs	2147119	60264029	60250379	54354820	**Buffer**	0.039					

Patient-16											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	92009450	64508813	57107667	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	63302217	64508813	57107667	**Buffer**	0.000					
5 mins	20673722	71271582	64508813	57107667	**Plasma**		0.328	100	3.843	100	96.16
5mins	675751	60615594	64508813	57107667	**Buffer**	0.013					
10 mins	28210401	71933288	64508813	57107667	**Plasma**		0.443	100	4.041	100	95.96
10 mins	974145	61466491	64508813	57107667	**Buffer**	0.018					
15 mins	32374253	67962957	64508813	57107667	**Plasma**		0.538	100	3.770	100	96.23
15 mins	1083599	60339650	64508813	57107667	**Buffer**	0.020					
20 mins	29110080	68180434	64508813	57107667	**Plasma**		0.482	100	4.732	100	95.27
20 mins	1213040	60042226	64508813	57107667	**Buffer**	0.023					
30 mins	35358739	67455422	64508813	57107667	**Plasma**		0.592	100	3.546	100	96.45
30 mins	1154177	62090777	64508813	57107667	**Buffer**	0.021					
1 hrs	34654586	69283717	64508813	57107667	**Plasma**		0.565	100	3.683	100	96.32
1 hrs	1117993	60688211	64508813	57107667	**Buffer**	0.021					

4 hrs	31444564	61290757	64508813	57107667	**Plasma**		0.580	100	2.462	100	97.54
4 hrs	727990	57632425	64508813	57107667	**Buffer**	0.014					
12 hrs	36542580	63867653	64508813	57107667	**Plasma**		0.646	100	2.785	100	97.21
12 hrs	935098	58673163	64508813	57107667	**Buffer**	0.018					
24 hrs	62415029	60660099	64508813	57107667	**Plasma**		1.162	100	2.601	100	97.40
24 hrs	1541038	57578489	64508813	57107667	**Buffer**	0.030					

Patient-17											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
5 mins	11816625	15027654	13936966	11905302	**Plasma**		0.921	100	2.610	100	97.39
5mins	285824	13924998	13936966	11905302	**Buffer**	0.024					
10 mins	11721749	16637312	13936966	11905302	**Plasma**		0.825	100	2.384	100	97.62
10 mins	250966	14941228	13936966	11905302	**Buffer**	0.020					
15 mins	13090494	16653499	13936966	11905302	**Plasma**		0.920	100	1.829	100	98.17
15 mins	222258	15457914	13936966	11905302	**Buffer**	0.017					
20 mins	13420303	17598351	13936966	11905302	**Plasma**		0.893	100	1.715	100	98.29
20 mins	207513	15867596	13936966	11905302	**Buffer**	0.015					

25 mins	13630674	17223815	13936966	11905302	**Plasma**		0.926	100	2.064	100	97.94
25 mins	267096	16350103	13936966	11905302	**Buffer**	0.019					
30 mins	13737716	17327993	13936966	11905302	**Plasma**		0.928	100	2.191	100	97.81
30 mins	284923	16402795	13936966	11905302	**Buffer**	0.020					
1 hrs	12599769	17733039	13936966	11905302	**Plasma**		0.832	100	1.902	100	98.10
1 hrs	222391	16457064	13936966	11905302	**Buffer**	0.016					
4 hrs	10185092	16738683	13936966	11905302	**Plasma**		0.712	100	1.695	100	98.30
4 hrs	172194	16699156	13936966	11905302	**Buffer**	0.012					
12 hrs	13231151	16321085	13936966	11905302	**Plasma**		0.949	100	2.207	100	97.79
12 hrs	302516	16905677	13936966	11905302	**Buffer**	0.021					
24 hrs	20081329	16760492	13936966	11905302	**Plasma**		1.403	100	0.456	100	99.54
24 hrs	89679	16412036	13936966	11905302	**Buffer**	0.006					

Patient-18											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	18055421	16244092	14544621	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	16980966	16244092	14544621	**Buffer**	0.000					
5 mins	2778703	18276398	16244092	14544621	**Plasma**		0.170	100	0.849	100	99.15

5mins	22549	17471234	16244092	14544621	**Buffer**	0.001					
10 mins	3964685	18440951	16244092	14544621	**Plasma**		0.240	100	1.169	100	98.83
10 mins	42025	16725064	16244092	14544621	**Buffer**	0.003					
15 mins	5692698	18157665	16244092	14544621	**Plasma**		0.350	100	1.190	100	98.81
15 mins	64726	17351401	16244092	14544621	**Buffer**	0.004					
20 mins	7688244	18746157	16244092	14544621	**Plasma**		0.458	100	2.346	100	97.65
20 mins	165692	17224467	16244092	14544621	**Buffer**	0.011					
25 mins	7316159	18541743	16244092	14544621	**Plasma**		0.441	100	3.730	100	96.27
25 mins	250451	17015945	16244092	14544621	**Buffer**	0.016					
30 mins	9430618	18466919	16244092	14544621	**Plasma**		0.570	100	3.781	100	96.22
30 mins	330080	17095639	16244092	14544621	**Buffer**	0.022					
1 hrs	11091403	17327257	16244092	14544621	**Plasma**		0.715	100	3.213	100	96.79
1 hrs	343145	16684126	16244092	14544621	**Buffer**	0.023					
4 hrs	13097803	16967389	16244092	14544621	**Plasma**		0.862	100	3.952	100	96.05
4 hrs	513661	16837274	16244092	14544621	**Buffer**	0.034					
12 hrs	13998292	16193854	16244092	14544621	**Plasma**		0.965	100	2.082	100	97.92
12 hrs	297044	16506808	16244092	14544621	**Buffer**	0.020					
24 hrs	23050877	16389252	16244092	14544621	**Plasma**		1.571	100	3.288	100	96.71

24 hrs	741292	16030397	16244092	14544621	**Buffer**	0.052					

Patient-19											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	16150458	16244092	14544621	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	16460365	16244092	14544621	**Buffer**	0.000					
5 mins	12064395	17243990	16244092	14544621	**Plasma**		0.781	100	2.604	100	97.40
5mins	280543	15396961	16244092	14544621	**Buffer**	0.020					
10 mins	14942564	16994735	16244092	14544621	**Plasma**		0.982	100	3.125	100	96.88
10 mins	430908	15681074	16244092	14544621	**Buffer**	0.031					
15 mins	15985642	16653724	16244092	14544621	**Plasma**		1.072	100	3.813	100	96.19
15 mins	590650	16137649	16244092	14544621	**Buffer**	0.041					
20 mins	15969623	16411384	16244092	14544621	**Plasma**		1.087	100	3.495	100	96.50
20 mins	534224	15708551	16244092	14544621	**Buffer**	0.038					
25 mins	15307860	16292590	16244092	14544621	**Plasma**		1.049	100	3.096	100	96.90
25 mins	462562	15902865	16244092	14544621	**Buffer**	0.032					
30 mins	14201779	15687458	16244092	14544621	**Plasma**		1.011	100	2.711	100	97.29
30 mins	384671	15675355	16244092	14544621	**Buffer**	0.027					

1 hrs	12994417	15662950	16244092	14544621	**Plasma**		0.927	100	3.076	100	96.92
1 hrs	389047	15243314	16244092	14544621	**Buffer**	0.029					
4 hrs	18342851	15410561	16244092	14544621	**Plasma**		1.329	100	3.766	100	96.23
4 hrs	671222	14973423	16244092	14544621	**Buffer**	0.050					
12 hrs	18009408	14923192	16244092	14544621	**Plasma**		1.348	100	1.428	100	98.57
12 hrs	265382	15396278	16244092	14544621	**Buffer**	0.019					
24 hrs	27110646	14991457	16244092	14544621	**Plasma**		2.020	100	2.557	100	97.44
24 hrs	741292	16030397	16244092	14544621	**Buffer**	0.052					

Patient-20											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	550025158	67824355	59849279	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	266478588	67824355	59849279	**Buffer**	0.000					
5 mins	19285344	63427914	67824355	59849279	**Plasma**		0.345	100	1.918	100	98.08
5mins	330319	56639274	67824355	59849279	**Buffer**	0.007					
10 mins	23585325	66400809	67824355	59849279	**Plasma**		0.403	100	2.256	100	97.74
10 mins	449019	56027565	67824355	59849279	**Buffer**	0.009					

15 mins	37752968	79218627	67824355	59849279	**Plasma**		0.540	100	3.204	100	96.80
15 mins	1069929	70076005	67824355	59849279	**Buffer**	0.017					
20 mins	32719182	78360762	67824355	59849279	**Plasma**		0.473	100	3.023	100	96.98
20 mins	874232	69253733	67824355	59849279	**Buffer**	0.014					
25 mins	31315916	79738410	67824355	59849279	**Plasma**		0.445	100	5.028	100	94.97
25 mins	1408105	71304210	67824355	59849279	**Buffer**	0.022					
30 mins	28685657	79885362	67824355	59849279	**Plasma**		0.407	100	2.964	100	97.04
30 mins	753073	70749175	67824355	59849279	**Buffer**	0.012					
1 hrs	31140815	79202873	67824355	59849279	**Plasma**		0.446	100	2.487	100	97.51
1 hrs	675505	69080605	67824355	59849279	**Buffer**	0.011					
4 hrs	54587294	72857239	67824355	59849279	**Plasma**		0.849	100	2.530	100	97.47
4 hrs	1335463	70439823	67824355	59849279	**Buffer**	0.021					
12 hrs	77751061	70567219	67824355	59849279	**Plasma**		1.249	100	2.326	100	97.67
12 hrs	1769569	69047391	67824355	59849279	**Buffer**	0.029					
24 hrs	100709689	67343522	67824355	59849279	**Plasma**		1.695	100	1.487	100	98.51
24 hrs	1531323	68847792	67824355	59849279	**Buffer**	0.025					

Patient-21											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	408128640	67824355	59849279	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	179042648	67824355	59849279	**Buffer**	0.000					
5 mins	42519121	79250742	67824355	59849279	**Plasma**		0.608	100	1.767	100	98.23
5mins	658429	69456602	67824355	59849279	**Buffer**	0.011					
10 mins	69732609	79681581	67824355	59849279	**Plasma**		0.992	100	1.870	100	98.13
10 mins	1101921	67323040	67824355	59849279	**Buffer**	0.019					
15 mins	75255986	78884478	67824355	59849279	**Plasma**		1.081	100	2.138	100	97.86
15 mins	1475317	72321635	67824355	59849279	**Buffer**	0.023					
20 mins	82403201	81305159	67824355	59849279	**Plasma**		1.149	100	1.858	100	98.14
20 mins	1305245	69315315	67824355	59849279	**Buffer**	0.021					
25 mins	82627126	79635504	67824355	59849279	**Plasma**		1.176	100	1.991	100	98.01
25 mins	1427712	69109251	67824355	59849279	**Buffer**	0.023					
30 mins	80951208	78076863	67824355	59849279	**Plasma**		1.175	100	2.167	100	97.83
30 mins	1553919	69162248	67824355	59849279	**Buffer**	0.025					
1 hrs	98358369	76764903	67824355	59849279	**Plasma**		1.452	100	2.097	100	97.90

1 hrs	1837299	68384380	67824355	59849279	**Buffer**	0.030					
4 hrs	105493602	73214337	67824355	59849279	**Plasma**		1.633	100	2.698	100	97.30
4 hrs	2628091	67608090	67824355	59849279	**Buffer**	0.044					
12 hrs	115068454	70132132	67824355	59849279	**Plasma**		1.859	100	2.921	100	97.08
12 hrs	3149241	65715905	67824355	59849279	**Buffer**	0.054					
24 hrs	140756934	67343582	67824355	59849279	**Plasma**		2.369	100	2.244	100	97.76
24 hrs	3155870	67293646	67824355	59849279	**Buffer**	0.053					

Patient-22											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	97861903	79804896	58429984	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	80397147	79804896	58429984	**Buffer**	0.000					
5 mins	13774964	84899895	79804896	58429984	**Plasma**		0.222	100	2.987	100	97.01
5mins	370356	76426472	79804896	58429984	**Buffer**	0.007					
10 mins	35787523	81921354	79804896	58429984	**Plasma**		0.597	100	2.911	100	97.09
10 mins	953222	74948048	79804896	58429984	**Buffer**	0.017					
15 mins	39702428	82005908	79804896	58429984	**Plasma**		0.661	100	3.348	100	96.65
15 mins	1196232	73800489	79804896	58429984	**Buffer**	0.022					

20 mins	44547855	80650614	79804896	58429984	**Plasma**		0.754	100	3.198	100	96.80
20 mins	1275297	72196994	79804896	58429984	**Buffer**	0.024					
25 mins	45128653	80950612	79804896	58429984	**Plasma**		0.761	100	2.927	100	97.07
25 mins	1177140	72134876	79804896	58429984	**Buffer**	0.022					
30 mins	46435394	80849423	79804896	58429984	**Plasma**		0.784	100	3.663	100	96.34
30 mins	1487444	70697394	79804896	58429984	**Buffer**	0.029					
1 hrs	37478835	79095037	79804896	58429984	**Plasma**		0.647	100	2.930	100	97.07
1 hrs	995122	71667118	79804896	58429984	**Buffer**	0.019					
4 hrs	60793022	77811258	79804896	58429984	**Plasma**		1.067	100	3.012	100	96.99
4 hrs	1592785	67691797	79804896	58429984	**Buffer**	0.032					

Patient-23											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	61872720	54200634	46050821	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	56816191	54200634	46050821	**Buffer**	0.000					
5 mins	32269695	59948115	54200634	46050821	**Plasma**		0.634	100	4.299	100	95.70
5mins	1317238	56928218	54200634	46050821	**Buffer**	0.027					

10 mins	44065376	62958607	54200634	46050821	**Plasma**		0.824	100	4.899	100	95.10
10 mins	1957601	57092085	54200634	46050821	**Buffer**	0.040					
15 mins	48646460	60612173	54200634	46050821	**Plasma**		0.945	100	4.637	100	95.36
15 mins	2129765	57228282	54200634	46050821	**Buffer**	0.044					
20 mins	47010487	62223233	54200634	46050821	**Plasma**		0.889	100	4.929	100	95.07
20 mins	2115622	56815226	54200634	46050821	**Buffer**	0.044					
25 mins	48241127	60274797	54200634	46050821	**Plasma**		0.942	100	4.788	100	95.21
25 mins	2129050	55561829	54200634	46050821	**Buffer**	0.045					
30 mins	45536557	61998269	54200634	46050821	**Plasma**		0.864	100	4.718	100	95.28
30 mins	1959347	56541261	54200634	46050821	**Buffer**	0.041					
1 hrs	40511934	61024528	54200634	46050821	**Plasma**		0.781	100	3.813	100	96.19
1 hrs	1450585	57302802	54200634	46050821	**Buffer**	0.030					
4 hrs	40278732	61672897	54200634	46050821	**Plasma**		0.769	100	3.395	100	96.60
4 hrs	1245984	56200003	54200634	46050821	**Buffer**	0.026					
12 hrs	46907531	58301190	54200634	46050821	**Plasma**		0.947	100	5.223	100	94.78
12 hrs	2380591	56653576	54200634	46050821	**Buffer**	0.049					
24 hrs	84341870	57098311	54200634	46050821	**Plasma**		1.739	100	2.872	100	97.13
24 hrs	2394788	56440151	54200634	46050821	**Buffer**	0.050					

Patient-24											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	142851048	47666461	40642346	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	75242407	47666461	40642346	**Buffer**	0.000					
5 mins	23607879	68175789	47666461	40642346	**Plasma**		0.406	100	4.963	100	95.04
5mins	1019111	59298598	47666461	40642346	**Buffer**	0.020					
10 mins	24785547	74829236	47666461	40642346	**Plasma**		0.388	100	4.066	100	95.93
10 mins	792977	58872831	47666461	40642346	**Buffer**	0.016					
15 mins	28482166	77130425	47666461	40642346	**Plasma**		0.433	100	4.962	100	95.04
15 mins	1107226	60432453	47666461	40642346	**Buffer**	0.021					
20 mins	32481806	72907717	47666461	40642346	**Plasma**		0.523	100	5.423	100	94.58
20 mins	1466397	60693301	47666461	40642346	**Buffer**	0.028					
25 mins	32655354	73797991	47666461	40642346	**Plasma**		0.519	100	5.405	100	94.60
25 mins	1457129	60928514	47666461	40642346	**Buffer**	0.028					
30 mins	38727794	70093397	47666461	40642346	**Plasma**		0.648	100	4.451	100	95.55
30 mins	1447793	58870278	47666461	40642346	**Buffer**	0.029					
1 hrs	43514532	72046299	47666461	40642346	**Plasma**		0.708	100	4.363	100	95.64

1 hrs	1554821	59002367	47666461	40642346	**Buffer**	0.031					
4 hrs	51884228	59159384	47666461	40642346	**Plasma**		1.029	100	4.031	100	95.97
4 hrs	1956108	55326788	47666461	40642346	**Buffer**	0.041					
12 hrs	62991610	55599807	47666461	40642346	**Plasma**		1.329	100	3.518	100	96.48
12 hrs	2223514	55787290	47666461	40642346	**Buffer**	0.047					
24 hrs	69950550	54461775	47666461	40642346	**Plasma**		1.506	100	2.699	100	97.30
24 hrs	1913935	55213398	47666461	40642346	**Buffer**	0.041					

Patient-25											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	140236653	46802206	40444494	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	68146815	46802206	40444494	**Buffer**	0.000					
5 mins	23345186	62314909	46802206	40444494	**Plasma**		0.434	100	3.561	100	96.44
5mins	724957	54343731	46802206	40444494	**Buffer**	0.015					
10 mins	37896410	65796612	46802206	40444494	**Plasma**		0.667	100	4.800	100	95.20
10 mins	1569895	56782758	46802206	40444494	**Buffer**	0.032					
15 mins	42252800	66019449	46802206	40444494	**Plasma**		0.741	100	5.944	100	94.06
15 mins	2168563	57000498	46802206	40444494	**Buffer**	0.044					

20 mins	43356758	62755167	46802206	40444494	**Plasma**		0.799	100	6.145	100	93.85
20 mins	2404803	56646302	46802206	40444494	**Buffer**	0.049					
25 mins	42880643	65054273	46802206	40444494	**Plasma**		0.763	100	5.786	100	94.21
25 mins	2142708	56183092	46802206	40444494	**Buffer**	0.044					
30 mins	38298405	62077580	46802206	40444494	**Plasma**		0.714	100	4.934	100	95.07
30 mins	1650799	54225981	46802206	40444494	**Buffer**	0.035					
1 hrs	43453209	59287689	46802206	40444494	**Plasma**		0.848	100	4.199	100	95.80
1 hrs	1639621	53275534	46802206	40444494	**Buffer**	0.036					
4 hrs	35354527	52695989	46802206	40444494	**Plasma**		0.776	100	3.543	100	96.46
4 hrs	1228134	51664489	46802206	40444494	**Buffer**	0.028					
12 hrs	43074638	51439916	46802206	40444494	**Plasma**		0.969	100	4.107	100	95.89
12 hrs	1797223	52260386	46802206	40444494	**Buffer**	0.040					
24 hrs	72530342	50909984	46802206	40444494	**Plasma**		1.649	100	3.991	100	96.01
24 hrs	2821625	49621410	46802206	40444494	**Buffer**	0.066					

Patient-27											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	132365482	48085240	37171146	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	73292067	48085240	37171146	**Buffer**	0.000					
5 mins	11838100	51479569	48085240	37171146	**Plasma**		0.297	100	5.626	100	94.37
5mins	615144	47543639	48085240	37171146	**Buffer**	0.017					
10 mins	23031752	54593803	48085240	37171146	**Plasma**		0.546	100	6.484	100	93.52
10 mins	1321859	48324452	48085240	37171146	**Buffer**	0.035					
15 mins	27922693	52445985	48085240	37171146	**Plasma**		0.689	100	7.459	100	92.54
15 mins	1880500	47352929	48085240	37171146	**Buffer**	0.051					
20 mins	32027104	52540185	48085240	37171146	**Plasma**		0.789	100	7.287	100	92.71
20 mins	2123260	47800749	48085240	37171146	**Buffer**	0.057					
25 mins	35177157	53471385	48085240	37171146	**Plasma**		0.851	100	7.017	100	92.98
25 mins	2326352	50394917	48085240	37171146	**Buffer**	0.060					
30 mins	38662992	51739398	48085240	37171146	**Plasma**		0.967	100	7.265	100	92.73
30 mins	2595352	47807063	48085240	37171146	**Buffer**	0.070					
1 hrs	42514900	50570419	48085240	37171146	**Plasma**		1.088	100	6.328	100	93.67

1 hrs	2513229	47243566	48085240	37171146	**Buffer**	0.069					
4 hrs	42108613	47593547	48085240	37171146	**Plasma**		1.145	100	4.875	100	95.12
4 hrs	1991376	46173992	48085240	37171146	**Buffer**	0.056					
12 hrs	53497361	45973490	48085240	37171146	**Plasma**		1.505	100	3.889	100	96.11
12 hrs	2077987	45920753	48085240	37171146	**Buffer**	0.059					
24 hrs	71617216	46231817	48085240	37171146	**Plasma**		2.004	100	3.040	100	96.96
24 hrs	2159772	45866230	48085240	37171146	**Buffer**	0.061					

Patient-28											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	108412923	108792486	76914663	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	95600924	108792486	76914663	**Buffer**	0.000					
5 mins	35694022	108062324	108792486	76914663	**Plasma**		0.467	100	2.915	100	97.09
5mins	942915	97916072	108792486	76914663	**Buffer**	0.014					
10 mins	86364661	110572696	108792486	76914663	**Plasma**		1.105	100	3.912	100	96.09
10 mins	3151794	103137337	108792486	76914663	**Buffer**	0.043					
15 mins	92511149	110619245	108792486	76914663	**Plasma**		1.183	100	4.293	100	95.71
15 mins	3585160	99857560	108792486	76914663	**Buffer**	0.051					

20 mins	110233121	104041543	108792486	76914663	**Plasma**		1.499	100	4.376	100	95.62
20 mins	4473852	96488114	108792486	76914663	**Buffer**	0.066					
25 mins	114007610	110052265	108792486	76914663	**Plasma**		1.465	100	4.313	100	95.69
25 mins	4432977	99206426	108792486	76914663	**Buffer**	0.063					
30 mins	123461260	112613990	108792486	76914663	**Plasma**		1.551	100	4.465	100	95.53
30 mins	4913006	100366328	108792486	76914663	**Buffer**	0.069					
1 hrs	134494662	106656585	108792486	76914663	**Plasma**		1.784	100	4.407	100	95.59
1 hrs	5656058	101770223	108792486	76914663	**Buffer**	0.079					
4 hrs	101159749	104901691	108792486	76914663	**Plasma**		1.364	100	3.380	100	96.62
4 hrs	3232669	99174042	108792486	76914663	**Buffer**	0.046					
12 hrs	101567124	103424150	108792486	76914663	**Plasma**		1.389	100	2.809	100	97.19
12 hrs	2740854	99370252	108792486	76914663	**Buffer**	0.039					
24 hrs	96680459	93782896	108792486	76914663	**Plasma**		1.458	100	1.912	100	98.09
24 hrs	2054732	104227062	108792486	76914663	**Buffer**	0.028					

Patient-29											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				

Baseline	0	105492134	90624685	69171709	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	99976728	90624685	69171709	**Buffer**	0.000					
5 mins	154069689	103633808	90624685	69171709	**Plasma**		1.948	100	1.172	100	98.83
5mins	1703315	97756771	90624685	69171709	**Buffer**	0.023					
10 mins	194052596	102439682	90624685	69171709	**Plasma**		2.482	100	1.332	100	98.67
10 mins	2520292	99847147	90624685	69171709	**Buffer**	0.033					
15 mins	184024731	100401671	90624685	69171709	**Plasma**		2.401	100	1.181	100	98.82
15 mins	2192194	101309584	90624685	69171709	**Buffer**	0.028					
20 mins	171479444	104181687	90624685	69171709	**Plasma**		2.156	100	1.220	100	98.78
20 mins	1979628	98546202	90624685	69171709	**Buffer**	0.026					
25 mins	171888980	107000668	90624685	69171709	**Plasma**		2.105	100	1.367	100	98.63
25 mins	2274783	103583739	90624685	69171709	**Buffer**	0.029					
30 mins	166691469	105384266	90624685	69171709	**Plasma**		2.072	100	1.431	100	98.57
30 mins	2250847	99464104	90624685	69171709	**Buffer**	0.030					
1 hrs	186229354	105027299	90624685	69171709	**Plasma**		2.323	100	1.237	100	98.76
1 hrs	2216888	101061703	90624685	69171709	**Buffer**	0.029					
4 hrs	229698883	98560762	90624685	69171709	**Plasma**		3.053	100	1.286	100	98.71
4 hrs	2919643	97452016	90624685	69171709	**Buffer**	0.039					
12 hrs	216585518	102384518	90624685	69171709	**Plasma**		2.771	100	1.252	100	98.75

12 hrs	2643249	99808606	90624685	69171709	**Buffer**	0.035					
24 hrs	63178791	107054705	90624685	69171709	**Plasma**		0.773	100	0.111	100	99.89
24 hrs	64383	98483353	90624685	69171709	**Buffer**	0.001					

Patient-30											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	109412923	108792486	76914663	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	100186484	108792486	76914663	**Buffer**	0.000					
5 mins	103014856	104005099	108792486	76914663	**Plasma**		1.401	100	2.934	100	97.07
5mins	2896083	99669926	108792486	76914663	**Buffer**	0.041					
10 mins	126668348	108840880	108792486	76914663	**Plasma**		1.646	100	4.189	100	95.81
10 mins	4941814	101374814	108792486	76914663	**Buffer**	0.069					
15 mins	144688780	107275183	108792486	76914663	**Plasma**		1.908	100	3.837	100	96.16
15 mins	5218054	100826193	108792486	76914663	**Buffer**	0.073					
20 mins	138132017	104631542	108792486	76914663	**Plasma**		1.867	100	3.900	100	96.10
20 mins	5098497	99017536	108792486	76914663	**Buffer**	0.073					
25 mins	138055965	105363129	108792486	76914663	**Plasma**		1.853	100	3.696	100	96.30
25 mins	4989489	103028324	108792486	76914663	**Buffer**	0.068					

30 mins	134814656	106776354	108792486	76914663	**Plasma**		1.786	100	3.802	100	96.20
30 mins	4902905	102129790	108792486	76914663	**Buffer**	0.068					
1 hrs	131759429	105046458	108792486	76914663	**Plasma**		1.774	100	2.968	100	97.03
1 hrs	3657617	98242457	108792486	76914663	**Buffer**	0.053					
4 hrs	122884760	99956060	108792486	76914663	**Plasma**		1.739	100	2.717	100	97.28
4 hrs	3359801	100574425	108792486	76914663	**Buffer**	0.047					
24 hrs	59740709	102858903	108792486	76914663	**Plasma**		0.822	100	0.558	100	99.44
24 hrs	325299	100430694	108792486	76914663	**Buffer**	0.005					

Patient-31											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	133835936	108792486	76914663	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	107730124	108792486	76914663	**Buffer**	0.000					
5 mins	52753985	107204314	108792486	76914663	**Plasma**		0.696	100	2.423	100	97.58
5mins	1199463	100586771	108792486	76914663	**Buffer**	0.017					
10 mins	47393576	104278618	108792486	76914663	**Plasma**		0.643	100	2.026	100	97.97
10 mins	916954	99569581	108792486	76914663	**Buffer**	0.013					
15 mins	93609404	103661414	108792486	76914663	**Plasma**		1.277	100	2.705	100	97.30
15 mins	2453414	100430750	108792486	76914663	**Buffer**	0.035					

20 mins	92847807	105786203	108792486	76914663	**Plasma**		1.241	100	2.810	100	97.19
20 mins	2524991	102393147	108792486	76914663	**Buffer**	0.035					
25 mins	89520408	106588062	108792486	76914663	**Plasma**		1.188	100	3.176	100	96.82
25 mins	2717991	101906223	108792486	76914663	**Buffer**	0.038					
30 mins	93038472	105452461	108792486	76914663	**Plasma**		1.248	100	3.192	100	96.81
30 mins	2824392	100303560	108792486	76914663	**Buffer**	0.040					
1 hrs	88910619	103462537	108792486	76914663	**Plasma**		1.216	100	2.619	100	97.38
1 hrs	2338762	103917510	108792486	76914663	**Buffer**	0.032					
4 hrs	91398882	103925056	108792486	76914663	**Plasma**		1.244	100	3.232	100	96.77
4 hrs	2914246	102528535	108792486	76914663	**Buffer**	0.040					
12 hrs	1.11E+08	104091384	108792486	76914663	**Plasma**		1.511	100	3.411	100	96.59
12 hrs	3623462	99411282	108792486	76914663	**Buffer**	0.052					
24 hrs	91250109	102254033	108792486	76914663	**Plasma**		1.262	100	2.635	100	97.37
24 hrs	2351427	99982803	108792486	76914663	**Buffer**	0.033					

Hip Joint surgery Patients

Patient-1-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	145046268	120401195	931829169	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	148946420	120401195	931829169	**Buffer**	0.000					
5 mins	92581040	146627448	120401195	931829169	**Plasma**		0.816	100	2.466	100	97.53
5mins	2178032	139883847	120401195	931829169	**Buffer**	0.020					
10 mins	118849242	143133064	120401195	931829169	**Plasma**		1.073	100	2.616	100	97.38
10 mins	3002762	138221570	120401195	931829169	**Buffer**	0.028					
15 mins	125132724	183820138	120401195	931829169	**Plasma**		0.880	100	1.895	100	98.105
15 mins	1806624	140082711	120401195	931829169	**Buffer**	0.017					
20 mins	97272271	167944008	120401195	931829169	**Plasma**		0.748	100	2.642	100	97.36
20 mins	2199116	143704873	120401195	931829169	**Buffer**	0.020					
25 mins	103560368	142947059	120401195	931829169	**Plasma**		0.936	100	2.688	100	97.31
25 mins	2811464	144392987	120401195	931829169	**Buffer**	0.025					
30 mins	121231697	141414102	120401195	931829169	**Plasma**		1.108	100	2.449	100	97.55
30 mins	2926199	139375636	120401195	931829169	**Buffer**	0.027					

1 hrs	110361645	141157893	120401195	931829169	**Plasma**		1.010	100	2.435	100	97.57
1 hrs	2704708	142064301	120401195	931829169	**Buffer**	0.025					
4 hrs	157282662	120513049	120401195	931829169	**Plasma**		1.686	100	1.245	100	98.75
4 hrs	2235856	137552999	120401195	931829169	**Buffer**	0.021					
24 hrs	111348661	155828809	120401195	931829169	**Plasma**		0.923	100	1.506	100	98.49
24 hrs	1487745	138255950	120401195	931829169	**Buffer**	0.014					

Patient-2-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc.in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	165958584	124376818	981016852	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	145098549	124376818	981016852	**Buffer**	0.000					
5 mins	152414278	134114488	124376818	981016852	**Plasma**		1.441	100	2.900	100	97.10
5mins	4883251	148174904	124376818	981016852	**Buffer**	0.042					
10 mins	153144032	136167642	124376818	981016852	**Plasma**		1.426	100	3.150	100	96.85
10 mins	5109249	144198834	124376818	981016852	**Buffer**	0.045					
15 mins	133480363	134080254	124376818	981016852	**Plasma**		1.262	100	3.762	100	96.24
15 mins	5574119	148848192	124376818	981016852	**Buffer**	0.047					

20 mins	147206109	129617901	124376818	981016852	**Plasma**		1.440	100	3.653	100	96.35
20 mins	6154241	148361501	124376818	981016852	**Buffer**	0.053					
25 mins	148839570	137393365	124376818	981016852	**Plasma**		1.373	100	3.423	100	96.58
25 mins	5477727	147707398	124376818	981016852	**Buffer**	0.047					
30 mins	149365827	129239982	124376818	981016852	**Plasma**		1.465	100	3.505	100	96.50
30 mins	5979579	147612200	124376818	981016852	**Buffer**	0.051					
1 hrs	151729652	123706219	124376818	981016852	**Plasma**		1.555	100	2.411	100	97.59
1 hrs	4378574	148036619	124376818	981016852	**Buffer**	0.037					
4 hrs	135866031	122658354	124376818	981016852	**Plasma**		1.404	100	2.044	100	97.96
4 hrs	3266115	144258118	124376818	981016852	**Buffer**	0.029					
24 hrs	98021123	122624458	124376818	981016852	**Plasma**		1.013	100	1.445	100	98.55
24 hrs	1666962	144311503	124376818	981016852	**Buffer**	0.015					

Patient-3-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	151766862	124376818	981016852	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	149607946	124376818	981016852	**Buffer**	0.000					
5 mins	68888699	142507069	124376818	981016852	**Plasma**		0.613	100	4.024	100	95.98

5mins	2909880	149582240	124376818	981016852	**Buffer**	0.025					
10 mins	72212372	130975813	124376818	981016852	**Plasma**		0.699	100	3.645	100	96.36
10 mins	2984415	148515231	124376818	981016852	**Buffer**	0.025					
15 mins	128192057	131348839	124376818	981016852	**Plasma**		1.237	100	2.734	100	97.27
15 mins	3962296	148488435	124376818	981016852	**Buffer**	0.034					
20 mins	119368266	132574036	124376818	981016852	**Plasma**		1.142	100	2.536	100	97.46
20 mins	3404001	149065043	124376818	981016852	**Buffer**	0.029					
25 mins	105783065	132830643	124376818	981016852	**Plasma**		1.010	100	2.772	100	97.23
25 mins	3334519	151049562	124376818	981016852	**Buffer**	0.028					
30 mins	132636598	130542375	124376818	981016852	**Plasma**		1.288	100	3.101	100	96.90
30 mins	4740895	150452094	124376818	981016852	**Buffer**	0.040					
1 hrs	133561779	129305547	124376818	981016852	**Plasma**		1.310	100	2.466	100	97.53
1 hrs	3934979	154505330	124376818	981016852	**Buffer**	0.032					
4 hrs	106044288	125592931	124376818	981016852	**Plasma**		1.070	100	1.663	100	98.34
4 hrs	2084192	148461908	124376818	981016852	**Buffer**	0.018					
24 hrs	69637852	120292435	124376818	981016852	**Plasma**		0.734	100	0.882	100	99.12
24 hrs	761203	149033284	124376818	981016852	**Buffer**	0.006					

Patient-4-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc.in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	103172797	73158147	564304665	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	83814802	73158147	564304665	**Buffer**	0.000					
5 mins	55252415	95091532	73158147	564304665	**Plasma**		0.753	100	2.547	100	97.45
5mins	1256056	84876415	73158147	564304665	**Buffer**	0.019					
10 mins	60600805	87996193	73158147	564304665	**Plasma**		0.893	100	2.100	100	97.90
10 mins	1167782	80747007	73158147	564304665	**Buffer**	0.019					
15 mins	76120771	82775857	73158147	564304665	**Plasma**		1.192	100	3.122	100	96.88
15 mins	2335178	81325968	73158147	564304665	**Buffer**	0.037					
20 mins	78534674	82456260	73158147	564304665	**Plasma**		1.235	100	3.432	100	96.57
20 mins	2738940	83787215	73158147	564304665	**Buffer**	0.042					
25 mins	81910113	81693165	73158147	564304665	**Plasma**		1.300	100	2.947	100	97.05
25 mins	2456710	83144185	73158147	564304665	**Buffer**	0.038					
30 mins	82917543	80980297	73158147	564304665	**Plasma**		1.327	100	1.903	100	98.10
30 mins	1606226	82438997	73158147	564304665	**Buffer**	0.025					
1 hrs	80028924	81399924	73158147	564304665	**Plasma**		1.275	100	2.090	100	97.91
1 hrs	1666927	81104361	73158147	564304665	**Buffer**	0.027					

4 hrs	78188801	82046570	73158147	564304665	**Plasma**		1.235	100	1.617	100	98.38
4 hrs	1264820	82056895	73158147	564304665	**Buffer**	0.020					
24 hrs	81165246	76334388	73158147	564304665	**Plasma**		1.378	100	1.294	100	98.71
24 hrs	1120167	81406254	73158147	564304665	**Buffer**	0.018					

Patient-5-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	212056519	97244426	708033334	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	129850844	97244426	708033334	**Buffer**	0.000					
5 mins	166825115	142920184	97244426	708033334	**Plasma**		1.603	100	2.394	100	97.61
5mins	3624545	129692846	97244426	708033334	**Buffer**	0.038					
10 mins	133114546	192503600	97244426	708033334	**Plasma**		0.950	100	3.461	100	96.54
10 mins	3326601	138990852	97244426	708033334	**Buffer**	0.033					
15 mins	169070972	148768718	97244426	708033334	**Plasma**		1.561	100	2.469	100	97.531
15 mins	3507966	125020673	97244426	708033334	**Buffer**	0.039					
20 mins	164544402	156028115	97244426	708033334	**Plasma**		1.448	100	2.668	100	97.33
20 mins	3596469	127801106	97244426	708033334	**Buffer**	0.039					
25 mins	167931398	160374238	97244426	708033334	**Plasma**		1.438	100	2.478	100	97.52
25 mins	3373983	130031562	97244426	708033334	**Buffer**	0.036					
30 mins	160389527	147340630	97244426	708033334	**Plasma**		1.495	100	2.355	100	97.64

30 mins	3311007	129171326	97244426	708033334	**Buffer**	0.035					
1 hrs	153290434	152269349	97244426	708033334	**Plasma**		1.383	100	2.217	100	97.78
1 hrs	2781963	124659223	97244426	708033334	**Buffer**	0.031					
4 hrs	134799864	143266572	97244426	708033334	**Plasma**		1.292	100	1.668	100	98.33
4 hrs	1949444	124203827	97244426	708033334	**Buffer**	0.022					
24 hrs	107110361	113279908	97244426	708033334	**Plasma**		1.299	100	0.951	100	99.05
24 hrs	1101421	122530807	97244426	708033334	**Buffer**	0.012					

Patient-6-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	104784530	111729233	111601420	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	101572297	111729233	111601420	**Buffer**	0.000					
5 mins	46334351	107380184	111729233	111601420	**Plasma**		0.432	100	2.701	100	97.30
5mins	1113443	95547817	111729233	111601420	**Buffer**	0.012					
10 mins	50707488	108660189	111729233	111601420	**Plasma**		0.467	100	2.767	100	97.23
10 mins	1242212	96190236	111729233	111601420	**Buffer**	0.013					
25 mins	52795568	118008632	111729233	111601420	**Plasma**		0.448	100	2.949	100	97.05
25 mins	1281215	97108562	111729233	111601420	**Buffer**	0.013					

30 mins	55471493	110173389	111729233	111601420	**Plasma**		0.504	100	1.347	100	98.65
30 mins	654422	96500819	111729233	111601420	**Buffer**	0.007					
1 hrs	45108585	113748115	111729233	111601420	**Plasma**		0.397	100	3.538	100	96.46
1 hrs	1377339	98171611	111729233	111601420	**Buffer**	0.014					
24 hrs	71171325	99754976	111729233	111601420	**Plasma**		0.714	100	1.059	100	98.94
24 hrs	762670	100932976	111729233	111601420	**Buffer**	0.008					

Patient-7-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	104945251	102302046	96705865	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	100044881	102302046	96705865	**Buffer**	0.000					
5 mins	78794816	110486353	102302046	96705865	**Plasma**		0.754	100	2.288	100	97.71
5mins	1680081	102976279	102302046	96705865	**Buffer**	0.017					
10 mins	94069703	109724226	102302046	96705865	**Plasma**		0.907	100	2.367	100	97.63
10 mins	2239917	110371364	102302046	96705865	**Buffer**	0.021					
15 mins	101071781	106653832	102302046	96705865	**Plasma**		1.003	100	2.612	100	97.39
15 mins	2537127	102514394	102302046	96705865	**Buffer**	0.026					

20 mins	109980775	110929670	102302046	96705865	**Plasma**		1.049	100	2.666	100	97.33
20 mins	2927448	110749147	102302046	96705865	**Buffer**	0.028					
25 mins	106619014	107736166	102302046	96705865	**Plasma**		1.047	100	2.826	100	97.17
25 mins	2992990	107036327	102302046	96705865	**Buffer**	0.030					
30 mins	117880935	111232789	102302046	96705865	**Plasma**		1.121	100	2.482	100	97.52
30 mins	2909757	110634704	102302046	96705865	**Buffer**	0.028					
1 hrs	103340671	106190926	102302046	96705865	**Plasma**		1.029	100	2.297	100	97.70
1 hrs	2479753	110929948	102302046	96705865	**Buffer**	0.024					
4 hrs	93107940	105695082	102302046	96705865	**Plasma**		0.932	100	1.245	100	98.76
4 hrs	1209352	110265519	102302046	96705865	**Buffer**	0.012					
24 hrs	111911791	105631466	102302046	96705865	**Plasma**		1.121	100	1.049	100	98.95
24 hrs	1234661	111095317	102302046	96705865	**Buffer**	0.012					

Patient-8-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	259862379	100769435	94843007	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	126505919	100769435	94843007	**Buffer**	0.000					
5 mins	47106078	92269642	100769435	94843007	**Plasma**		0.542	100	4.030	100	95.97

5mins	1853112	90059166	100769435	94843007	**Buffer**	0.022					
10 mins	61900513	102754731	100769435	94843007	**Plasma**		0.640	100	3.288	100	96.71
10 mins	1789889	90359846	100769435	94843007	**Buffer**	0.021					
15 mins	61913300	94957393	100769435	94843007	**Plasma**		0.693	100	3.032	100	96.97
15 mins	1770240	89533751	100769435	94843007	**Buffer**	0.021					
20 mins	59754822	92301549	100769435	94843007	**Plasma**		0.688	100	3.234	100	96.77
20 mins	1920056	91705901	100769435	94843007	**Buffer**	0.022					
25 mins	53967837	92798484	100769435	94843007	**Plasma**		0.618	100	4.097	100	95.90
25 mins	2142381	89914571	100769435	94843007	**Buffer**	0.025					
30 mins	66261355	92087985	100769435	94843007	**Plasma**		0.765	100	3.387	100	96.61
30 mins	2245271	92140147	100769435	94843007	**Buffer**	0.026					
1 hrs	47025364	90983135	100769435	94843007	**Plasma**		0.549	100	3.095	100	96.90
1 hrs	1437123	89826880	100769435	94843007	**Buffer**	0.017					
4 hrs	30298392	89894139	100769435	94843007	**Plasma**		0.358	100	2.519	100	97.481
4 hrs	779375	91792696	100769435	94843007	**Buffer**	0.009					
24 hrs	30152320	96274208	100769435	94843007	**Plasma**		0.333	100	1.037	100	98.96
24 hrs	302089	92998266	100769435	94843007	**Buffer**	0.003					

Patient-9-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	128261567	124025669	108168530	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	125799309	124025669	108168530	**Buffer**	0.000					
5 mins	154202417	131185315	124025669	108168530	**Plasma**		1.348	100	1.383	100	98.62
5mins	2033867	125096388	124025669	108168530	**Buffer**	0.019					
10 mins	147316952	129339251	124025669	108168530	**Plasma**		1.306	100	1.622	100	98.38
10 mins	2273493	123043844	124025669	108168530	**Buffer**	0.021					
15 mins	145959427	130766210	124025669	108168530	**Plasma**		1.280	100	1.477	100	98.52
15 mins	2068593	125457541	124025669	108168530	**Buffer**	0.019					
20 mins	161561884	134504807	124025669	108168530	**Plasma**		1.377	100	1.242	100	98.76
20 mins	1809292	121236922	124025669	108168530	**Buffer**	0.017					
25 mins	146688216	133043462	124025669	108168530	**Plasma**		1.264	100	1.438	100	98.56
25 mins	2015879	127187557	124025669	108168530	**Buffer**	0.018					
30 mins	153088176	133457848	124025669	108168530	**Plasma**		1.315	100	1.680	100	98.32
30 mins	2399987	124555663	124025669	108168530	**Buffer**	0.022					
1 hrs	154792155	130575440	124025669	108168530	**Plasma**		1.359	100	1.596	100	98.40

1 hrs	2299870	121522291	124025669	108168530	**Buffer**	0.022					
24 hrs	121260552	129549562	124025669	108168530	**Plasma**		1.073	100	0.816	100	99.18
24 hrs	940320	123117910	124025669	108168530	**Buffer**	0.009					

Patient-10-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	186600895	119014989	104688217	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	139859768	119014989	104688217	**Buffer**	0.000					
5 mins	99422442	148652646	119014989	104688217	**Plasma**		0.760	100	4.880	100	95.12
5mins	4319101	132339209	119014989	104688217	**Buffer**	0.037					
10 mins	97210953	151322276	119014989	104688217	**Plasma**		0.730	100	4.509	100	95.49
10 mins	3867340	133526102	119014989	104688217	**Buffer**	0.033					
15 mins	85195858	150281546	119014989	104688217	**Plasma**		0.644	100	4.518	100	95.48
15 mins	3405841	132986505	119014989	104688217	**Buffer**	0.029					
20 mins	93399149	148432025	119014989	104688217	**Plasma**		0.715	100	4.471	100	95.53
20 mins	3652543	129830907	119014989	104688217	**Buffer**	0.032					
25 mins	85554440	161798244	119014989	104688217	**Plasma**		0.601	100	4.718	100	95.28
25 mins	3251770	130349576	119014989	104688217	**Buffer**	0.028					

30 mins	86016728	144001792	119014989	104688217	**Plasma**		0.679	100	3.966	100	96.03
30 mins	2993473	126363600	119014989	104688217	**Buffer**	0.027					
1 hrs	106604366	161725532	119014989	104688217	**Plasma**		0.749	100	3.884	100	96.12
1 hrs	3276543	127975299	119014989	104688217	**Buffer**	0.029					
4 hrs	59393780	135122346	119014989	104688217	**Plasma**		0.500	100	2.166	100	97.83
4 hrs	1189771	124977444	119014989	104688217	**Buffer**	0.011					
24 hrs	38060581	133633807	119014989	104688217	**Plasma**		0.324	100	1.165	100	98.83
24 hrs	413837	124714666	119014989	104688217	**Buffer**	0.004					

Patient-11-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	135470824	111384600	114692280	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	117239619	111384600	114692280	**Buffer**	0.000					
5 mins	69690540	125109206	111384600	114692280	**Plasma**		0.541	100	3.186	100	96.81
5mins	2016787	113633392	111384600	114692280	**Buffer**	0.017					
10 mins	90854794	123074659	111384600	114692280	**Plasma**		0.717	100	3.635	100	96.37
10 mins	3069531	114376185	111384600	114692280	**Buffer**	0.026					

15 mins	97466735	115767082	111384600	114692280	**Plasma**		0.818	100	3.810	100	96.19
15 mins	3706963	115556172	111384600	114692280	**Buffer**	0.031					
20 mins	112869258	120449293	111384600	114692280	**Plasma**		0.910	100	3.743	100	96.26
20 mins	4065277	115915457	111384600	114692280	**Buffer**	0.034					
25 mins	109827916	121114831	111384600	114692280	**Plasma**		0.881	100	3.226	100	96.77
25 mins	3372943	115292829	111384600	114692280	**Buffer**	0.028					
30 mins	103501906	113515258	111384600	114692280	**Plasma**		0.885	100	3.284	100	96.72
30 mins	3438289	114842509	111384600	114692280	**Buffer**	0.029					
1 hrs	95913564	113782332	111384600	114692280	**Plasma**		0.819	100	2.722	100	97.28
1 hrs	2731076	119018562	111384600	114692280	**Buffer**	0.022					
4 hrs	68825342	115828300	111384600	114692280	**Plasma**		0.577	100	2.200	100	97.80
4 hrs	1554476	118902004	111384600	114692280	**Buffer**	0.013					
24 hrs	46170968	118265494	111384600	114692280	**Plasma**		0.379	100	0.180	100	99.82
24 hrs	79538	112949840	111384600	114692280	**Buffer**	0.001					

Patient-12-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	412556638	78622034	92644628	**Plasma**		0.000	100	0.000	100	0.000

Baseline	0	155452744	78622034	92644628	**Buffer**	0.000					
5 mins	132089966	123709056	78622034	92644628	**Plasma**		0.906	100	1.489	100	98.51
5mins	1805435	113552361	78622034	92644628	**Buffer**	0.013					
10 mins	104096420	121181054	78622034	92644628	**Plasma**		0.729	100	1.928	100	98.07
10 mins	1619065	97782295	78622034	92644628	**Buffer**	0.014					
15 mins	131182746	112117034	78622034	92644628	**Plasma**		0.993	100	1.121	100	98.88
15 mins	1276432	97298438	78622034	92644628	**Buffer**	0.011					
20 mins	128527829	112679624	78622034	92644628	**Plasma**		0.968	100	1.415	100	98.59
20 mins	1525863	94523302	78622034	92644628	**Buffer**	0.014					
25 mins	133712599	115219601	78622034	92644628	**Plasma**		0.985	100	1.333	100	98.67
25 mins	1567676	101338218	78622034	92644628	**Buffer**	0.013					
30 mins	127722113	125167280	78622034	92644628	**Plasma**		0.866	100	1.241	100	98.76
30 mins	1327115	104837269	78622034	92644628	**Buffer**	0.011					
1 hrs	120168396	110831671	78622034	92644628	**Plasma**		0.920	100	0.988	100	99.01
1 hrs	1058297	98796999	78622034	92644628	**Buffer**	0.009					
4 hrs	74861865	111334294	78622034	92644628	**Plasma**		0.571	100	0.691	100	99.31
4 hrs	489327	105292789	78622034	92644628	**Buffer**	0.004					
24 hrs	59899774	110520298	78622034	92644628	**Plasma**		0.460	100	0.136	100	99.86
24 hrs	76910	103980138	78622034	92644628	**Buffer**	0.001					

Patient-13-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	126701302	94681130	96350727	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	95214608	94681130	96350727	**Buffer**	0.000					
5 mins	84534041	99491211	94681130	96350727	**Plasma**		0.835	100	3.364	100	96.64
5mins	2664224	93210171	94681130	96350727	**Buffer**	0.028					
10 mins	91237093	99014075	94681130	96350727	**Plasma**		0.905	100	4.192	100	95.81
10 mins	3291816	85216921	94681130	96350727	**Buffer**	0.038					
15 mins	91687211	98223748	94681130	96350727	**Plasma**		0.917	100	4.427	100	95.57
15 mins	3716360	89927396	94681130	96350727	**Buffer**	0.041					
20 mins	91551817	100747975	94681130	96350727	**Plasma**		0.893	100	3.318	100	96.68
20 mins	2619303	86861440	94681130	96350727	**Buffer**	0.030					
25 mins	87143466	99991941	94681130	96350727	**Plasma**		0.856	100	3.331	100	96.67
25 mins	2519600	86785840	94681130	96350727	**Buffer**	0.029					
30 mins	81791825	104023483	94681130	96350727	**Plasma**		0.773	100	3.311	100	96.69
30 mins	2269179	87171692	94681130	96350727	**Buffer**	0.026					

1 hrs	72955405	107469673	94681130	96350727	**Plasma**		0.667	100	2.921	100	97.08
1 hrs	1773976	89454335	94681130	96350727	**Buffer**	0.019					
4 hrs	73488631	101084797	94681130	96350727	**Plasma**		0.714	100	2.706	100	97.29
4 hrs	1758077	89372421	94681130	96350727	**Buffer**	0.019					
24 hrs	48795203	88916627	94681130	96350727	**Plasma**		0.539	100	0.940	100	99.06
24 hrs	450640	87398748	94681130	96350727	**Buffer**	0.005					

Patient-14-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	89676470	75694560	71549876	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	80241130	75694560	71549876	**Buffer**	0.000					
5 mins	100936997	92766405	75694560	71549876	**Plasma**		1.151	100	2.891	100	97.11
5mins	2529846	80419113	75694560	71549876	**Buffer**	0.033					
10 mins	110071194	89911023	75694560	71549876	**Plasma**		1.295	100	3.012	100	96.99
10 mins	2990422	81101365	75694560	71549876	**Buffer**	0.039					
15 mins	113753262	90847419	75694560	71549876	**Plasma**		1.325	100	3.103	100	96.90
15 mins	3230712	83139541	75694560	71549876	**Buffer**	0.041					
20 mins	113598508	92313405	75694560	71549876	**Plasma**		1.302	100	2.680	100	97.32

20 mins	2772000	84049293	75694560	71549876	**Buffer**	0.035					
25 mins	117169248	92843146	75694560	71549876	**Plasma**		1.335	100	2.615	100	97.39
25 mins	2769636	83916390	75694560	71549876	**Buffer**	0.035					
30 mins	99552986	115074867	75694560	71549876	**Plasma**		0.915	100	3.105	100	96.90
30 mins	2430077	90477712	75694560	71549876	**Buffer**	0.028					
1 hrs	117876587	98775308	75694560	71549876	**Plasma**		1.263	100	2.595	100	97.40
1 hrs	2641018	85266536	75694560	71549876	**Buffer**	0.033					
4 hrs	102090054	88424929	75694560	71549876	**Plasma**		1.221	100	2.220	100	97.78
4 hrs	2140976	83539809	75694560	71549876	**Buffer**	0.027					
24 hrs	32542143	88843562	75694560	71549876	**Plasma**		0.388	100	1.117	100	98.88
24 hrs	344769	84231302	75694560	71549876	**Buffer**	0.004					

Patient-15-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	196661249	144929232	126238740	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	153445108	144929232	126238740	**Buffer**	0.000					
5 mins	26877275	151714317	144929232	126238740	**Plasma**		0.203	100	2.348	100	97.65
5mins	602791	144898885	144929232	126238740	**Buffer**	0.005					

10 mins	47840927	152699705	144929232	126238740	**Plasma**		0.360	100	2.355	100	97.64
10 mins	978497	132601328	144929232	126238740	**Buffer**	0.008					
15 mins	80546848	160590802	144929232	126238740	**Plasma**		0.576	100	2.675	100	97.32
15 mins	1971182	146896094	144929232	126238740	**Buffer**	0.015					
20 mins	83822637	152193397	144929232	126238740	**Plasma**		0.632	100	3.132	100	96.87
20 mins	2518037	145985695	144929232	126238740	**Buffer**	0.020					
25 mins	93368873	168245037	144929232	126238740	**Plasma**		0.637	100	3.087	100	96.91
25 mins	2592268	151320763	144929232	126238740	**Buffer**	0.020					
30 mins	96186780	166823622	144929232	126238740	**Plasma**		0.662	100	3.429	100	96.57
30 mins	2902038	146787927	144929232	126238740	**Buffer**	0.023					
1 hrs	117728698	152327528	144929232	126238740	**Plasma**		0.887	100	2.727	100	97.27
1 hrs	3046845	144587725	144929232	126238740	**Buffer**	0.024					
4 hrs	107137804	147787972	144929232	126238740	**Plasma**		0.832	100	2.239	100	97.76
4 hrs	2277614	140350153	144929232	126238740	**Buffer**	0.019					
24 hrs	65763331	139876454	144929232	126238740	**Plasma**		0.540	100	1.588	100	98.41
24 hrs	1060194	142010510	144929232	126238740	**Buffer**	0.009					

Patient-16-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	185046772	129542175	116135975	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	152930914	129542175	116135975	**Buffer**	0.000					
5 mins	153322461	176482698	129542175	116135975	**Plasma**		0.969	100	2.792	100	97.21
5mins	3895179	160605989	129542175	116135975	**Buffer**	0.027					
10 mins	142016428	176221116	129542175	116135975	**Plasma**		0.899	100	3.053	100	96.95
10 mins	3945117	160333697	129542175	116135975	**Buffer**	0.027					
15 mins	119969940	189896239	129542175	116135975	**Plasma**		0.705	100	3.150	100	96.85
15 mins	3274416	164558747	129542175	116135975	**Buffer**	0.022					
20 mins	126471113	198034281	129542175	116135975	**Plasma**		0.712	100	3.315	100	96.69
20 mins	3548971	167628034	129542175	116135975	**Buffer**	0.024					
25 mins	142172386	186999352	129542175	116135975	**Plasma**		0.848	100	2.824	100	97.18
25 mins	3514234	163684760	129542175	116135975	**Buffer**	0.024					
30 mins	146686985	196460208	129542175	116135975	**Plasma**		0.833	100	3.299	100	96.70
30 mins	3961121	160824887	129542175	116135975	**Buffer**	0.027					

1 hrs	158261997	187101557	129542175	116135975	**Plasma**		0.944	100	2.774	100	97.23
1 hrs	3770381	160689004	129542175	116135975	**Buffer**	0.026					
4 hrs	69227487	101966011	129542175	116135975	**Plasma**		0.757	100	2.141	100	97.86
4 hrs	1486301	102267523	129542175	116135975	**Buffer**	0.016					
24 hrs	109977151	165756875	129542175	116135975	**Plasma**		0.740	100	1.506	100	98.49
24 hrs	1611326	161261002	129542175	116135975	**Buffer**	0.011					

Patient-17-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	119216607	102026676	92861297	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	109322617	102026676	92861297	**Buffer**	0.000					
5 mins	99574701	111374191	102026676	92861297	**Plasma**		0.982	100	3.351	100	96.65
5mins	3125016	104322001	102026676	92861297	**Buffer**	0.033					
10 mins	105651250	110386492	102026676	92861297	**Plasma**		1.052	100	3.194	100	96.81
10 mins	3249568	106308454	102026676	92861297	**Buffer**	0.034					
15 mins	114513099	112846862	102026676	92861297	**Plasma**		1.115	100	3.099	100	96.90
15 mins	3419431	108736394	102026676	92861297	**Buffer**	0.035					
20 mins	111809744	106323485	102026676	92861297	**Plasma**		1.155	100	2.902	100	97.10

20 mins	3249567	106475500	102026676	92861297	**Buffer**	0.034					
25 mins	108387671	118495084	102026676	92861297	**Plasma**		1.005	100	2.932	100	97.07
25 mins	2898406	108057949	102026676	92861297	**Buffer**	0.029					
30 mins	108253712	123829551	102026676	92861297	**Plasma**		0.961	100	2.985	100	97.01
30 mins	2863252	109740486	102026676	92861297	**Buffer**	0.029					
1 hrs	109612102	125692863	102026676	92861297	**Plasma**		0.958	100	2.687	100	97.31
1 hrs	2591440	110573297	102026676	92861297	**Buffer**	0.026					
4 hrs	94358445	95752082	102026676	92861297	**Plasma**		1.083	100	1.558	100	98.44
4 hrs	1563005	101831372	102026676	92861297	**Buffer**	0.017					
24 hrs	69025259	93544075	102026676	92861297	**Plasma**		0.811	100	1.316	100	98.68
24 hrs	996176	102593089	102026676	92861297	**Buffer**	0.011					

Patient-18-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	93059920	96836102	91835870	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	97821247	96836102	91835870	**Buffer**	0.000					
5 mins	106015395	91772608	96836102	91835870	**Plasma**		1.218	100	2.740	100	97.26
5mins	3072969	97077604	96836102	91835870	**Buffer**	0.033					

10 mins	114115939	91639736	96836102	91835870	**Plasma**		1.313	100	2.920	100	97.08
10 mins	3408082	93723942	96836102	91835870	**Buffer**	0.038					
15 mins	107342541	88699855	96836102	91835870	**Plasma**		1.276	100	2.888	100	97.11
15 mins	3345126	95705805	96836102	91835870	**Buffer**	0.037					
20 mins	105948066	87853944	96836102	91835870	**Plasma**		1.272	100	2.939	100	97.06
20 mins	3288761	92776413	96836102	91835870	**Buffer**	0.037					
25 mins	101036103	87409892	96836102	91835870	**Plasma**		1.219	100	2.803	100	97.19
25 mins	3105997	95868469	96836102	91835870	**Buffer**	0.034					
30 mins	98951215	91549863	96836102	91835870	**Plasma**		1.140	100	2.599	100	97.40
30 mins	2670153	95035033	96836102	91835870	**Buffer**	0.030					
1 hrs	95591192	93534606	96836102	91835870	**Plasma**		1.078	100	2.364	100	97.64
1 hrs	2307275	95490317	96836102	91835870	**Buffer**	0.025					
4 hrs	80885660	88433885	96836102	91835870	**Plasma**		0.964	100	2.051	100	97.95
4 hrs	1784888	95129836	96836102	91835870	**Buffer**	0.020					
24 hrs	41770743	89617579	96836102	91835870	**Plasma**		0.491	100	1.221	100	98.78
24 hrs	542434	95313763	96836102	91835870	**Buffer**	0.006					

Patient-19-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**%Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	110704588	82706945	73349284	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	93471032	82706945	73349284	**Buffer**	0.000					
5 mins	56005563	113893364	82706945	73349284	**Plasma**		0.554	100	2.262	100	97.74
5mins	1102485	99132012	82706945	73349284	**Buffer**	0.013					
10 mins	87528181	106359811	82706945	73349284	**Plasma**		0.928	100	2.752	100	97.25
10 mins	2077038	91713986	82706945	73349284	**Buffer**	0.026					
15 mins	111854440	114517341	82706945	73349284	**Plasma**		1.101	100	2.280	100	97.72
15 mins	2025826	90948678	82706945	73349284	**Buffer**	0.025					
20 mins	101648591	110761222	82706945	73349284	**Plasma**		1.035	100	2.655	100	97.34
20 mins	2191319	89936126	82706945	73349284	**Buffer**	0.027					
25 mins	99819820	108598870	82706945	73349284	**Plasma**		1.036	100	2.870	100	97.13
25 mins	2224281	84305622	82706945	73349284	**Buffer**	0.030					
30 mins	101911850	114862171	82706945	73349284	**Plasma**		1.000	100	2.864	100	97.14
30 mins	2226773	87616067	82706945	73349284	**Buffer**	0.029					

1 hrs	105708050	109762764	82706945	73349284	**Plasma**		1.086	100	2.694	100	97.31
1 hrs	2511428	96800460	82706945	73349284	**Buffer**	0.029					
4 hrs	105615940	95661015	82706945	73349284	**Plasma**		1.245	100	1.826	100	98.17
4 hrs	1713036	84987996	82706945	73349284	**Buffer**	0.023					
24 hrs	10065238	141270046	82706945	73349284	**Plasma**		0.080	100	0.207	100	99.79
24 hrs	16190	109887577	82706945	73349284	**Buffer**	0.000					

Patient-20-H											
Time	**A**	**B**	**C**	**D**	**Chambers**	**Conc. in Buffer Chamber**	**Conc. in Plasma Chamber**	**100**	**% Free**	**100**	**% Bound**
						µg/ml	µg/ml				
Baseline	0	630606075	82706945	73349284	**Plasma**		0.000	100	0.000	100	0.000
Baseline	0	294227582	82706945	73349284	**Buffer**	0.000					
5 mins	99678413	112133802	82706945	73349284	**Plasma**		1.002	100	4.035	100	95.97
5mins	3348937	93374620	82706945	73349284	**Buffer**	0.040					
10 mins	82248456	127256153	82706945	73349284	**Plasma**		0.729	100	4.123	100	95.88
10 mins	2673551	100327543	82706945	73349284	**Buffer**	0.030					
15 mins	101813015	145335396	82706945	73349284	**Plasma**		0.790	100	4.539	100	95.46
15 mins	3236807	101785623	82706945	73349284	**Buffer**	0.036					

20 mins	96585327	147932832	82706945	73349284	**Plasma**		0.736	100	4.299	100	95.70
20 mins	2693567	95957372	82706945	73349284	**Buffer**	0.032					
25 mins	81413990	125618300	82706945	73349284	**Plasma**		0.731	100	3.905	100	96.10
25 mins	2386270	94287321	82706945	73349284	**Buffer**	0.029					
30 mins	73871543	91135966	82706945	73349284	**Plasma**		0.914	100	2.793	100	97.21
30 mins	1943883	85877969	82706945	73349284	**Buffer**	0.026					
1 hrs	101511897	110786305	82706945	73349284	**Plasma**		1.033	100	2.728	100	97.27
1 hrs	2472624	98922478	82706945	73349284	**Buffer**	0.028					

Appendix 2

Determination of AGP in plasma of patients

Hip joint surgery patients

1H	Unbound µg/ml	Bound µg/ml	AGP mg/100ml
Baseline	0.0000	0.0000	10.36
5 mins	0.0201	0.8158	13.84
10 mins	0.0281	1.073	14.42
15 mins	0.0167	0.8796	15.37
20 mins	0.0198	0.7484	14.38
25 mins	0.0252	0.9361	14.73
30 mins	0.0271	1.108	15.14
1 hrs	0.0246	1.010	15.10
4 hrs	0.0210	1.686	15.45
24 hrs	0.0139	0.9233	23.50

2H	Unbound µg/ml	Bound µg/ml	AGP mg/100ml
Baseline	0.0000	0.0000	8.881
5 mins	0.0418	1.441	17.03
10 mins	0.0449	1.426	17.36
15 mins	0.0475	1.262	17.28
20 mins	0.0526	1.440	16.58

25 mins	0.0470	1.374	16.29
30 mins	0.0514	1.465	17.30
1 hrs	0.0375	1.555	18.13
4 hrs	0.0287	1.404	18.72
24 hrs	0.0146	1.014	26.44

3H	Unbound µg/ml	Bound µg/ml	AGP mg/100ml
Baseline	0.0000	0.0000	5.190
5 mins	0.0247	0.6129	18.71
10 mins	0.0255	0.6990	14.81
15 mins	0.0338	1.237	17.27
20 mins	0.0290	1.142	18.44
25 mins	0.0280	1.010	17.98
30 mins	0.0400	1.288	18.31
1 hrs	0.0323	1.309	18.49
4 hrs	0.0178	1.071	21.01
24 hrs	0.0065	0.7340	27.04

4H	Unbound µg/ml	Bound µg/ml	AGP mg/100ml
Baseline	0.000	0.000	22.20
5 mins	0.0192	0.7533	20.65

10 mins	0.0187	0.8928	22.28
15 mins	0.0372	1.192	21.80
20 mins	0.0424	1.235	21.40
25 mins	0.0383	1.300	22.15
30 mins	0.0253	1.327	21.59
1 hrs	0.0266	1.275	21.58
4 hrs	0.0200	1.236	21.64
24 hrs	0.0178	1.379	32.77

5H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	14.79
5 mins	0.0384	1.603	22.17
10 mins	0.0329	0.9497	23.03
15 mins	0.0385	1.560	24.44
20 mins	0.0387	1.448	21.86
25 mins	0.0356	1.438	23.18
30 mins	0.0352	1.495	22.45
1 hrs	0.0307	1.383	22.66
4 hrs	0.0216	1.292	26.35
24 hrs	0.0123	1.299	29.36

6H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	22.22
5 mins	0.0117	0.4320	27.08
10 mins	0.0129	0.4672	24.60
25 mins	0.0132	0.4479	26.10
30 mins	0.0068	0.5041	27.19
1 hrs	0.0140	0.3970	28.21
24 hrs	0.0076	0.7143	42.06

7H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	23.05
5 mins	0.0173	0.7544	16.09
10 mins	0.0215	0.9069	17.84
15 mins	0.0262	1.002	14.68
20 mins	0.0280	1.049	14.83
25 mins	0.0296	1.047	15.04
30 mins	0.0278	1.121	14.27
1 hrs	0.0236	1.030	15.24
4 hrs	0.0116	0.9319	17.55
24 hrs	0.0118	1.121	20.54

8H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	11.73
5 mins	0.0219	0.5424	9.663
10 mins	0.0210	0.6401	10.00

15 mins	0.0210	0.6928	10.84
20 mins	0.0222	0.6878	10.39
25 mins	0.0253	0.6179	9.369
30 mins	0.0259	0.7645	9.678
1 hrs	0.0170	0.5492	10.20
4 hrs	0.0090	0.3581	9.445
24 hrs	0.0035	0.3328	13.23

9H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	14.36
5 mins	0.0186	1.348	9.986
10 mins	0.0212	1.306	12.13
15 mins	0.0189	1.280	10.87
20 mins	0.0171	1.377	9.789
25 mins	0.0182	1.264	10.60
30 mins	0.0221	1.315	12.03
1 hrs	0.0217	1.359	10.49
24 hrs	0.0088	1.073	16.57

10H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	8.553
5 mins	0.0371	0.7604	8.003
10 mins	0.0329	0.7303	7.651
15 mins	0.0291	0.6445	7.510
20 mins	0.0320	0.7154	7.516

25 mins	0.0284	0.6011	7.511
30 mins	0.0269	0.6791	7.522
1 hrs	0.0291	0.7494	7.628
4 hrs	0.0108	0.4997	7.740
24 hrs	0.0038	0.3238	9.094

11H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	9.841
5 mins	0.0172	0.5410	9.255
10 mins	0.0261	0.7169	9.180
15 mins	0.0312	0.8176	8.667
20 mins	0.0341	0.9100	8.960
25 mins	0.0284	0.8807	8.562
30 mins	0.0291	0.8855	9.033
1 hrs	0.0223	0.8186	8.069
4 hrs	0.0127	0.5771	8.541
24 hrs	0.0007	0.3791	14.52

12H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	22.51
5 mins	0.0135	0.9061	19.87
10 mins	0.0141	0.7290	20.87
15 mins	0.0111	0.9930	19.23
20 mins	0.0137	0.9680	19.49
25 mins	0.0131	0.9848	19.71

30 mins	0.0107	0.8660	20.41
1 hrs	0.0091	0.9201	21.66
4 hrs	0.0039	0.5706	20.93
24 hrs	0.0006	0.4599	23.42

13H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	10.04
5 mins	0.0281	0.8349	9.221
10 mins	0.0380	0.9055	9.795
15 mins	0.0406	0.9173	9.717
20 mins	0.0296	0.8930	9.369
25 mins	0.0285	0.8564	9.201
30 mins	0.0256	0.7727	9.210
1 hrs	0.0195	0.6671	8.796
4 hrs	0.0193	0.7144	9.960
24 hrs	0.0051	0.5393	14.36

14H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	16.37
5 mins	0.0333	1.151	15.03
10 mins	0.0390	1.295	14.53
15 mins	0.0411	1.325	14.10
20 mins	0.0349	1.302	14.46
25 mins	0.0349	1.335	14.26
30 mins	0.0284	0.9152	13.86

1 hrs	0.0328	1.262	14.70
4 hrs	0.0271	1.221	14.52
24 hrs	0.0043	0.3875	20.38

15H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	12.17
5 mins	0.0048	0.2034	11.47
10 mins	0.0085	0.3597	10.98
15 mins	0.0154	0.5758	10.93
20 mins	0.0198	0.6323	10.32
25 mins	0.0197	0.6371	11.30
30 mins	0.0227	0.6619	11.76
1 hrs	0.0242	0.8873	11.00
4 hrs	0.0186	0.8323	12.34
24 hrs	0.0086	0.5398	16.09

16H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	10.53
5 mins	0.0271	0.9691	9.054
10 mins	0.0274	0.8989	8.993
15 mins	0.0222	0.7047	8.963
20 mins	0.0236	0.7124	8.229
25 mins	0.0239	0.8480	8.255
30 mins	0.0275	0.8328	8.458
1 hrs	0.0262	0.9435	8.950

4 hrs	0.0162	0.7573	15.43
24 hrs	0.0111	0.7401	12.52

17H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	21.56
5 mins	0.0329	0.9823	16.00
10 mins	0.0336	1.052	17.09
15 mins	0.0346	1.115	16.25
20 mins	0.0335	1.155	16.65
25 mins	0.0295	1.005	16.60
30 mins	0.0287	0.9605	17.20
1 hrs	0.0257	0.9581	17.46
4 hrs	0.0169	1.083	18.90
24 hrs	0.0107	0.8107	24.98

18H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	13.07
5 mins	0.0334	1.218	13.36
10 mins	0.0383	1.313	13.06
15 mins	0.0369	1.276	12.86
20 mins	0.0374	1.272	12.70
25 mins	0.0342	1.219	12.42
30 mins	0.0296	1.140	11.31
1 hrs	0.0255	1.078	13.14
4 hrs	0.0198	0.9644	14.51

24 hrs	0.0060	0.4915	20.81

19H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	19.56
5 mins	0.0125	0.5545	18.96
10 mins	0.0255	0.9279	17.68
15 mins	0.0251	1.101	17.20
20 mins	0.0275	1.035	18.08
25 mins	0.0297	1.036	16.79
30 mins	0.0287	1.000	16.19
1 hrs	0.0293	1.086	16.04
4 hrs	0.0227	1.245	15.84
24 hrs	0.0002	0.0803	22.18

20H	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	17.19
5 mins	0.0404	1.002	14.01
10 mins	0.0300	0.7288	14.45
15 mins	0.0359	0.7899	15.10
20 mins	0.0317	0.7362	14.63
25 mins	0.0285	0.7308	14.58
30 mins	0.0255	0.9140	15.69
1 hrs	0.0282	1.033	15.52

Knee joint surgery patients

Patient 1 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	19.45
5 mins	0.0182	0.7820	19.43
10 mins	0.0184	1.044	18.64
15 mins	0.0210	0.9313	17.99
20 mins	0.0196	0.8602	20.10
25 mins	0.0161	0.8590	20.70
30 mins	0.0151	0.8444	19.99
1 hrs	0.0142	0.8385	20.21
4 hrs	0.0084	0.7473	21.50
24 hrs	0.0100	0.9608	20.05
Pre-topup-1	0.0119	0.8984	15.13
Pre-topup-2	0.0140	1.064	17.67
Pre-topup-3	0.0138	0.9881	18.01
Post-topup-2	0.0146	1.104	14.82
Post-topup-3	0.0141	0.8407	15.50

Patient 2 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	9.940
5 mins	0.0493	1.216	9.613
10 mins	0.0608	1.361	9.543
15 mins	0.0483	1.276	9.719
20 mins	0.0436	1.182	9.340
25 mins	0.0378	1.051	9.354

30 mins	0.0400	1.016	9.615
1 hrs	0.0305	0.8739	9.270
4 hrs	0.0224	0.5896	9.339
24 hrs	0.0221	0.7915	14.04
Pre-topup-1	0.0222	0.6645	9.439
Pre-topup-2	0.0259	0.7434	9.271
Pre-topup-3	0.0250	0.7098	10.53
Post-topup-1	0.0215	0.7484	7.877
Post-topup-2	0.0303	0.8729	7.604
Post-topup-3	0.0235	0.7719	10.84
Pre-Rescue-1	0.0375	0.6988	8.942
Post-Rescue-1	0.0406	0.7554	8.511

Patient 3 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	16.43
5 mins	0.0302	1.313	14.69
10 mins	0.0307	1.275	14.42
15 mins	0.0036	1.434	14.06
20 mins	0.0284	1.359	14.17
25 mins	0.0337	1.370	14.64
30 mins	0.0288	1.265	14.52
1 hrs	0.0217	1.244	14.87
4 hrs	0.0314	1.171	14.32
24 hrs	0.0300	1.803	16.95
Pre-topup-1	0.0286	1.213	13.74

Pre-topup-2	0.0307	1.522	15.54
Pre-topup-3	0.0243	1.476	11.05
Post-topup-1	0.0268	1.623	11.00
Post-topup-2	0.0312	1.884	11.22
Post-topup-3	0.0209	1.686	12.40

Patient 4 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	16.53
5 mins	0.0280	1.174	16.59
10 mins	0.0305	1.440	16.99
15 mins	0.0381	1.631	16.55
20 mins	0.0386	1.535	14.01
25 mins	0.0377	1.445	15.79
30 mins	0.0326	1.483	16.47
1 hr	0.0292	1.360	16.62
24 hr	0.0053	0.7052	21.57
Pre top up 1	0.0193	0.8851	16.22
Pre top up 2	0.0156	0.9209	9.943
Pre top up 3	0.0098	0.7152	19.05
Post top up 1	0.0188	1.043	12.81
Post top up 2	0.0153	0.8026	13.43
Post top up 3	0.0052	1.006	14.26

Patient 5 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	12.45

5 mins	0.0139	0.6432	11.61
10 mins	0.0168	0.8058	11.13
15 mins	0.0222	1.006	10.29
20 mins	0.0279	1.111	9.755
25 mins	0.0330	1.186	9.368
30 mins	0.0361	1.288	10.86
1 hr	0.0317	1.380	11.65
24 hr	0.0229	1.619	13.07
Pre top up 2	0.0336	1.394	11.04
Pre top up 3	0.0169	1.037	12.08
Post top up 1	0.0288	1.268	10.26
Post top up 3	0.0212	1.571	8.421

Patient 6 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	15.10
5 mins	0.0314	0.8303	11.27
10 mins	0.0441	1.046	11.40
15 mins	0.0393	0.9909	11.97
20 mins	0.0375	0.9681	10.96
25 mins	0.0360	0.9262	11.50
30 mins	0.0431	1.027	12.59
1 hr	0.0388	0.9305	12.31
4hrs	0.0313	1.199	14.17
24 hr	0.0465	1.810	16.75
Pre top up 1	0.0372	1.300	14.78

Pre top up 2	0.0270	1.370	16.42
Pre top up 3	0.0363	1.440	12.16
Post top up 1	0.0434	1.610	10.13
Post top up 2	0.0542	1.510	11.30
Post top up 3	0.0500	1.776	12.01

Patient 7 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	28.04
5 mins	0.0159	0.7343	26.46
10 mins	0.0269	0.9142	26.91
15 mins	0.0250	1.153	25.61
20 mins	0.0270	1.113	26.83
25 mins	0.0308	1.232	27.17
30 mins	0.0258	1.130	26.61
1 hr	0.0253	1.160	28.96
4hrs	0.0109	0.8794	29.80
24 hr	0.0068	1.107	35.44
Pre top up 1	0.0092	0.7831	29.69
Pre top up 2	0.0086	0.7165	29.50
Pre top up 3	0.0019	0.6174	26.71
Post top up 1	0.0120	0.8815	24.08
Post top up 3	0.0103	1.366	28.01

Patient 8 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	24.56

5 mins	0.0501	1.792	26.37
10 mins	0.0563	1.775	29.68
15 mins	0.0597	2.196	30.26
20 mins	0.0718	2.305	30.45
25 mins	0.0625	2.160	30.82
30 mins	0.0573	2.137	29.81
1 hr	0.0446	1.937	31.01
24 hr	0.0021	0.3427	36.49
Pre top up 1	0.0216	1.212	31.28
Pre top up 2	0.0179	0.943	28.59
Pre top up 3	0.0062	0.6141	35.92
Post top up 1	0.0268	1.420	26.90
Post top up 2	0.0163	0.8571	34.14
Post top up 3	0.0024	0.5397	32.72

Patient 9 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	17.35
5 mins	0.0283	0.9249	18.80
10 mins	0.0311	1.083	18.94
15 mins	0.0325	1.131	17.50
20 mins	0.0304	1.132	19.42
25 mins	0.0324	1.151	17.81
30 mins	0.0346	1.160	18.20
1 hr	0.0235	1.012	18.88
24 hr	0.0221	1.424	24.06

Pre top up 1	0.0172	0.8709	18.99
Pre top up 2	0.0155	0.9709	19.77
Pre top up 3	0.0283	1.061	19.91
Post top up 1	0.0205	1.154	15.20
Post top up 2	0.0234	0.9099	16.77
Post top up 3	0.0325	1.438	17.43

Patient 10 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	24.39
5 mins	0.0125	0.2298	20.36
10 mins	0.0229	0.9172	19.49
15 mins	0.0281	1.199	19.96
20 mins	0.0276	1.228	19.60
25 mins	0.0239	1.247	19.64
30 mins	0.0248	1.304	19.26
1 hr	0.0259	1.239	20.63
24 hr	0.0237	1.680	24.70
Pre top up 1	0.0257	1.089	16.96
Pre top up 3	0.0148	0.8699	22.38
Post top up 1	0.0313	1.551	19.01
Post top up 3	0.0241	1.452	23.51

Patient 11 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	31.20
5 mins	0.0129	0.8251	24.05

10 mins	0.0149	0.8374	24.30
15 mins	0.0138	0.9602	25.26
20 mins	0.0142	1.033	24.25
25 mins	0.0156	1.084	25.18
30 mins	0.0161	1.156	23.81
1 hr	0.0142	1.138	25.16
24 hr	0.0103	1.589	33.90
Pre top up 1	0.0177	1.338	25.65
Pre top up 2	0.0189	2.092	30.49
Pre top up 3	0.0090	1.221	34.31
Post top up 1	0.0170	1.533	20.76
Post top up 2	0.0347	2.337	21.70
Post top up 3	0.0101	1.535	29.13

Patient 12 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	20.13
5 mins	0.0077	0.1337	15.30
10 mins	0.0157	0.4455	14.79
15 mins	0.0299	0.8006	14.98
20 mins	0.0329	0.9198	14.63
25 mins	0.0345	0.9807	14.63
30 mins	0.0286	0.8968	14.38
1 hr	0.0400	1.370	16.35
24 hr	0.0212	1.135	21.06
Pre top up 1	0.0314	1.172	16.49

Post top up 1	0.0367	1.570	17.67

Patient 13 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	25.21
5 mins	0.0161	0.2465	20.45
10 mins	0.0157	0.4487	20.32
15 mins	0.0183	0.5295	20.74
20 mins	0.0206	0.5647	20.25
25 mins	0.0171	0.5809	19.84
30 mins	0.0205	0.7167	20.60
1 hr	0.0301	1.149	21.27
24 hr	0.0377	1.779	28.32
Pre top up 1	0.0441	1.667	23.14
Pre top up 2	0.0480	2.034	27.51
Post top up 1	0.0419	1.909	23.38
Post top up 2	0.0407	2.112	21.33

Patient 14 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	14.81
5 mins	0.0135	0.5021	11.90
10 mins	0.0239	0.7958	12.24
15 mins	0.0292	0.9426	11.77
20 mins	0.0329	1.101	11.36
25 mins	0.0343	1.030	12.10
30 mins	0.0361	1.050	11.73

1 hr	0.0329	1.067	12.08
24 hr	0.0258	1.294	14.42
Pre top up 1	0.0320	1.171	13.17
Pre top up 2	0.0321	1.352	12.04
Pre top up 3	0.0352	1.180	14.12
Post top up 1	0.0309	1.127	9.746
Post top up 2	0.0325	1.081	9.601
Post top up 3	0.0352	1.098	9.600

Patient 15 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	21.02
5 mins	0.0101	0.4872	18.10
10 mins	0.0112	0.5247	17.31
15 mins	0.0119	0.5253	16.72
20 mins	0.0131	0.6419	17.77
25 mins	0.0145	0.7109	14.77
30 mins	0.0232	0.9167	15.86
1 hr	0.0323	1.158	15.74
24 hr	0.0158	1.058	17.85
Pre top up 1	0.0297	1.280	17.50
Pre top up 2	0.0326	1.517	19.39
Pre top up 3	0.0201	1.182	17.82
Post top up 1	0.0384	1.687	13.74
Post top up 2	0.0300	1.621	14.45
Post top up 3	0.0176	1.103	14.11

Patient 16 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	15.40
5 mins	0.0225	0.9772	12.66
10 mins	0.0647	1.524	13.69
15 mins	0.0487	1.777	13.19
20 mins	0.0585	1.937	13.28
25 mins	0.0578	1.921	12.09
30 mins	0.0614	1.889	12.87
1 hr	0.0644	2.030	13.67
24 hr	0.0041	0.5744	21.01
Pre top up 1	0.0314	1.528	14.29
Pre top up 2	0.0273	1.4370	15.41
Pre top up 3	0.0206	0.8076	19.27
Post top up 1	0.0310	1.412	8.622
Post top up 2	0.0256	1.646	12.616
Post top up 3	0.0019	0.1286	15.205

Patient 17 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	16.97
5 mins	0.0330	0.9471	13.63
10 mins	0.0494	1.349	13.49
15 mins	0.0463	2.155	14.10
20 mins	0.0447	1.271	13.04
25 mins	0.0476	1.300	13.27

30 mins	0.0480	1.308	11.12
1 hr	0.0410	1.359	12.21
24 hr	0.0360	2.128	17.31
Pre top up 1	0.0410	1.817	10.96
Pre top up 2	0.0501	1.939	14.11
Pre top up 3	0.0306	2.027	14.11
Post top up 1	0.0746	2.102	13.30
Post top up 2	0.0536	2.006	11.81
Post top up 3	0.0075	0.4093	13.88

Patient 18 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	57.07
5 mins	0.0042	1.374	54.72
10 mins	0.0100	2.120	52.08
15 mins	0.0132	1.492	55.63
20 mins	0.0124	2.131	61.37
25 mins	0.0090	2.163	54.33
30 mins	0.0306	2.397	59.72
1 hr	0.0111	2.042	59.72
24 hr	0.0024	1.632	51.93
Pre top up 1	0.0135	2.883	48.23
Pre top up 2	0.0204	3.182	58.80
Pre top up 3	0.0044	1.369	66.49
Post top up 1	0.0180	4.000	40.58
Post top up 2	0.0191	2.665	49.92

Post top up 3	0.0010	0.4410	52.47

Patient 19 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	16.45
5 mins	0.0027	0.2439	17.56
10 mins	0.0137	0.4653	18.51
15 mins	0.0137	0.4693	19.49
20 mins	0.0183	0.6720	17.99
25 mins	0.0245	0.7982	20.28
30 mins	0.0234	0.7878	19.99
1 hr	0.0302	1.046	20.32
24 hr	0.0320	1.453	21.56
Pre top up 1	0.0354	1.279	20.20
Pre top up 3	0.0242	1.260	23.18
Post top up 1	0.0436	1.479	18.82
Post top up 3	0.0069	0.2854	16.55

Patient 20 K	Unbound µg/ml	Bound µg/ml	AGP mg/100mL
Baseline	0.0000	0.0000	32.19
5 mins	0.0156	0.5767	29.28
10 mins	0.0227	0.7899	30.42
15 mins	0.0335	1.017	31.18
20 mins	0.0368	1.147	27.46
25 mins	0.0386	1.198	29.49
30 mins	0.0385	1.313	27.35

1 hr	0.0373	1.366	26.12
Pre top up 1	0.0286	1.487	33.30
Pre top up 2	0.0499	1.943	37.44
Pre top up 3	0.0376	1.664	32.15
Post top up 1	0.0445	1.683	30.08
Post top up 3	0.0102	0.4303	25.63