

**The *in vitro* and *in vivo* Formation and Potency of 6 $\beta$ -Naltrexol,  
the Major Human Metabolite of Naltrexone**

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## Abstract

6 $\beta$ -Naltrexol is the major human metabolite of naltrexone, which is an opioid receptor antagonist used in the treatment of opioid and alcohol dependence. This metabolite is thought to contribute to the pharmacological effects of naltrexone, particularly the longer duration of naltrexone compared to naloxone (the prototypical opioid receptor antagonist), but to what extent has not been fully described.

6 $\beta$ -Naltrexol was synthesised from naltrexone in order to conduct the studies contained in this thesis as it was not commercially available at the time. Additionally, a validated HPLC assay method needed to be developed to quantify naltrexone and 6 $\beta$ -naltrexol for the *in vivo* and *in vitro* studies contained within. 6 $\beta$ -Naltrexol was successfully synthesised, and the HPLC assay was developed for simultaneous analysis of the parent and metabolite in a number of biological fluids, and performed with a high degree of precision and accuracy throughout.

The enzyme kinetics for the formation of 6 $\beta$ -naltrexol from naltrexone were determined *in vitro* in human liver cytosolic and microsomal preparations. Additionally, several compounds were tested for their likelihood of inhibition of this formation. The hepatic enzymatic formation of 6 $\beta$ -naltrexol from naltrexone was confined to the cytosolic and not the microsomal fraction, exhibited considerable intersubject variability and could be inhibited by a number of compounds. The most potent of these were certain steroid hormones, and naloxone.

The *in vivo* pharmacokinetics and bioavailability of naltrexone, and the formation of 6 $\beta$ -naltrexol, were also assessed after oral and intravenous administration of naltrexone to healthy volunteers. Naltrexone and 6 $\beta$ -naltrexol were quantified in the plasma, urine and saliva of these subjects. Additionally, the correlation between 6 $\beta$ -naltrexol concentrations and increased subjective side-effects reported previously was assessed. As with the *in vitro* studies, there was a high degree of interindividual variation of pharmacokinetic parameters. It was found that saliva is possibly a better alternative to plasma in assessing naltrexone status following the 50 mg dose used clinically. There was no correlation between high biofluid concentrations of 6 $\beta$ -naltrexol and an increase in subjective side effects after intravenous or oral naltrexone administration.

Potency studies and assessment of the duration of antagonistic activity of 6 $\beta$ -naltrexol were conducted *in vitro* in electrically-stimulated guinea pig ileum preparations (blocking the

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morphine-induced twitch height) and *in vivo* in mice (reversing morphine-induced antinociception). The potencies were compared to the parent naltrexone, and naloxone. Naltrexone was more potent than naloxone in the guinea pig ileum preparation and interestingly, 6 $\beta$ -naltrexol was found to be 4.5-fold more potent than naloxone, and nearly three times more potent than naltrexone in this preparation. The high potency found in the *in vitro* study was not reflected in the *in vivo* mouse study, in which 6 $\beta$ -naltrexol showed only 1/185<sup>th</sup> the potency of naltrexone. Whereas the *in vivo* potency of 6 $\beta$ -naltrexol was much lower than that of naltrexone or naloxone, the duration of action was much longer.

The *in vivo* potency of 6 $\beta$ -naltrexol is lower than that of its parent compound naltrexone, but the longer duration of action, and the significantly higher plasma concentrations of this metabolite after an oral dose of naltrexone indicate that 6 $\beta$ -naltrexol will contribute significantly to the therapeutic effects of naltrexone.

## Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Susan Porter and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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### List of abbreviations used in this thesis

The abbreviations and prefixes of the International System of units have been used in this thesis, except for the alternatives listed below. Additional abbreviations and terminology, and pharmacokinetic symbols are also listed.

AKR	aldo-keto reductase family of enzymes
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the concentration-time curve
cAMP	cyclic 3',5'-adenosine monophosphate
CL	total systemic clearance
CL <sub>int</sub>	intrinsic clearance
CL/F	apparent oral clearance
CL <sub>R</sub>	apparent renal clearance
C <sub>max</sub>	maximum measured concentration
CNS	central nervous system
COMT	catechol -O-methyltransferase
CV	coefficient of variation (expressed as a percentage)
CYP450	cytochrome P450 enzyme
DADLE	D-Ala- $\Delta$ -leu-enkephalin
DAMGO	[D-Ala <sup>2</sup> ,N-MePhe <sup>4</sup> ,Gly-ol <sup>5</sup> ]enkephalin
DD	dihydrodiol dehydrogenase
DSLET	[D-Ser <sup>2</sup> ,Leu <sup>5</sup> ,Thr <sup>6</sup> ]enkephalin
DRG	dorsal root ganglion
DSM-IV	Diagnostic and Statistical Manual (Volume IV)
ECD	electrochemical detection
EC <sub>50</sub>	effective concentration eliciting 50% of maximal effect
ED <sub>50</sub>	effective dose eliciting 50% of maximal effect
fu	fraction unbound in plasma
GC	gas chromatography
GPCR	guanine nucleotide binding (G) -protein coupled receptor
HEK	human embryonic kidney
HMN	2-hydroxy-3-methoxynaltrexone
HPLC	high pressure (performance) liquid chromatography
HQC	high quality control
IC <sub>50</sub>	concentration of antagonist that inhibits agonist action by 50%
ID <sub>50</sub>	dose of antagonist that inhibits agonist action by 50%
icv	intracerebroventricular
im	intramuscular
ip	intraperitoneal
it	intrathecal
IUPHAR	International Union of Pharmacologists
IV	intravenous
K <sub>A</sub>	equilibrium dissociation constant of a drug for its receptor
K <sub>i</sub>	inhibition constant
K <sub>m</sub>	affinity constant of enzyme for substrate, concentration at which reaction is half of V <sub>max</sub> (Michaelis-Menten dissociation constant)
LOQ	limit of quantification
LQC	low quality control
M6G	morphine-6-glucuronide
MQC	medium quality control
MS	mass spectrometry
MSC	Methadone Symptoms Checklist

MW	molecular weight
n	number within a sample
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	nuclear magnetic resonance
pA <sub>2</sub>	measure of affinity of an antagonist to its receptor (-log K <sub>B</sub> )
PFPA	pentafluoropropionic acid
PK	pharmacokinetic
pKa	acidity constant log <sub>10</sub> transformed (pH at which 50% of the compound is ionised)
POMS	Profile of Mood States
QC	quality control
r	correlation coefficient
r <sup>2</sup>	coefficient of determination
Rf	retention factor
RI	reference interval
rpm	revolutions per minute
sc	subcutaneous
SD	standard deviation
SEM	standard error of the mean
<sup>35</sup> S-GTPγS	<sup>35</sup> S-guanosine triphosphate-gamma S
SNP	single nucleotide polymorphism
t <sub>1/2</sub>	half-life
tlc	thin layer chromatography
Tmax	time at which maximum concentration is achieved
UV	ultraviolet
V	rate of formation of substrate
V <sub>d</sub>	apparent volume of distribution
V <sub>max</sub>	maximum reaction velocity

## 1. Naltrexone and 6 $\beta$ -Naltrexol: A Review of the Literature

### 1.1. Summary

Naltrexone is an opioid antagonist, which has been used extensively in opioid overdose and dependence (Weinrieb and O'Brien, 1997), and alcohol dependence (Anton, 1996). 6 $\beta$ -Naltrexol is the major human metabolite of naltrexone. Naltrexone is a synthetic compound, first synthesised in 1963 (Blumberg and Dayton, 1974), and is an antagonist with higher affinity for the mu opioid receptor than for either delta or kappa opioid receptors. The use of naltrexone increased markedly in the last decade of the 20<sup>th</sup> century, particularly in relation to conditions related to the “reward pathways” of the central nervous system (CNS), such as dependencies, eating disorders and obsessive behaviours.

Despite the increased use of naltrexone, there are still substantial gaps in our knowledge of the basic pharmacokinetics and pharmacodynamics of this drug, and the formation and activity of an active metabolite in humans (6 $\beta$ -naltrexol), with a much longer duration of action than the parent compound (Chatterjee et al., 1974). There is a paucity of data relating to this metabolite which is the reduced product of naltrexone. This could be due, in part, to the non-commercial availability of 6 $\beta$ -naltrexol. The factors controlling the metabolism of naltrexone to this metabolite, including the possible effects of long-term treatment with naltrexone on opioid receptor function and bodily functions are also largely unknown.

### 1.2. Opioid Pharmacology

#### 1.2.1. History

Humans have been using the dried juice from the unripe seed of the opium poppy *Papaver somniferum* for medicinal reasons and pleasure for thousands of years. The ancient Sumerians (inhabiting what is now Iraq) and Egyptians recognised its analgesic and antidiarrhoeal properties. The Sumerians called opium “gil” meaning joy; thus its euphoric properties were well known to them. Such notables of history and medicine as Hippocrates ( $\approx$ 460 BC), Dioscorides (AD 60) and Galen (131 AD), discussed the therapeutic properties of the plant (Brownstein, 1993).

For centuries the mood-altering effects, particularly euphoria, of opium have been recognised, particularly by the Turks, Iranians and Arabians, who introduced it to the Indians and Chinese, where it has been traditionally taken orally. The spread of opium into all parts of Europe from Asia Minor occurred between the tenth and thirteenth centuries, and with it addiction (Brownstein, 1993).

Sixteenth century manuscripts have been found describing abuse and tolerance to opium in Turkey, Egypt, Germany and England. From the late seventeenth to the mid-nineteenth centuries, opium was consumed among the higher social stratum in British Society mainly as the oral preparation ‘tincture of opium’, or laudanum. The problem of dependence also became widespread in China (one of the biggest producers of the drug), where the efforts to suppress the sale and use of opium failed as the British, later joined by the French, forced the Chinese to permit opium trade and consumption (Brownstein, 1993).

Morphine, the principle active component of opium, (see Figure 1.1) accounts for approximately 10% of juice, and was first isolated in 1806 by Friedrich Sertürner in Paderborn, Germany. This was followed by the isolation of codeine (0.5% of juice) in 1832, and papaverine (1.0% of juice) in 1848 by Merck (Jaffe and Martin, 1992). When the hypodermic needle and syringe were invented in the mid-nineteenth century, the use of morphine became more widespread, and opium dependence became even more prevalent. Morphine proved to have as much abuse potential as opium, which led to the search for and synthesis of drugs with potentially less abuse potential, fewer side effects (such as respiratory depression), and higher analgesic potency. One such compound was diacetylmorphine (heroin) synthesised in 1898, which was claimed to be “as potent as morphine and free from abuse liability” (Brownstein, 1993). This has proven not to be the case, and studies relating to the treatment of dependence of heroin are prolific. Morphine remains the standard against which new analgesics are compared.

Various compounds with substitutions at the nitrogen, and carbons 3, 6, and 14 (see morphine structure Figure 1.1), were synthesised from the 1920’s onwards (e.g. oxycodone), and shown to have agonist effects such as analgesia, pupil constriction, decreased gastrointestinal motility, depression of respiration and cough reflex and euphoria. Nalorphine was the first antagonist synthesised (in 1941) and could reverse the respiratory depression caused by morphine and as such was a theoretically useful blocker of the potentially lethal effects of respiratory depression. However, this compound showed some agonist properties, such as analgesia, respiratory depression and psychotomimetic reactions (Blumberg and Dayton, 1974). Cyclazocine was also shown to be a potent antagonist (Martin et al., 1973), but produced dysphoria, making it an unattractive option for patients (Resnick et al., 1974). Blumberg and Dayton, in 1961 tested the N-allyl derivative of oxymorphone, naloxone (Figure 1.1) which was shown to be at least 10 times more potent an antagonist than

nalorphine in animals. This finding led to the synthesis and investigation of other derivatives, one of which, EN1639 or naltrexone (Figure 1.1), was eight times more potent than naloxone in blocking the oxymorphone-induced loss of righting reflex in rats. In addition, the duration of action of naltrexone was three times that of naloxone with only one eighth of the dose (Blumberg and Dayton, 1974). Furthermore, in mice, naltrexone was three times more potent than naloxone in counteracting the oxymorphone-induced Straub tail, as discussed below with ED<sub>50</sub> values of 0.047 and 0.14 mg/kg for naltrexone and naloxone, respectively (Blumberg and Dayton, 1974). The preclinical pharmacology of naltrexone is discussed more fully in 1.3.3.1.

With the cloning of the opioid receptor genes in the 1990s, and the discovery of a number of polymorphisms of these genes since, it is envisaged that targeted treatments for conditions such as pain and dependence, that involve activation of the opioid receptors, will emerge.

### **1.2.2. Opioid Structure**

Apart from the opium alkaloids, and their semi-synthetic derivatives, which fall into the phenanthrene or benzyloisoquinoline classes, a number of other chemical classes of drugs have pharmacological actions similar to morphine. These include the morphinans; benzomorphanes, such as pentazocine; phenylpiperidines, such as pethidine; and diphenylheptylamines including methadone.

Regarding the phenanthrene opioid agonists, substitutions of the phenolic hydroxyl at C3 with a bulkier group, as in heroin (acetyl) and codeine (methyl) markedly reduces the binding affinity to opioid receptors whereas the most obvious difference between the agonists and antagonists is the greater degree of substitution on the nitrogen for the antagonists as shown in Figure 1.1.

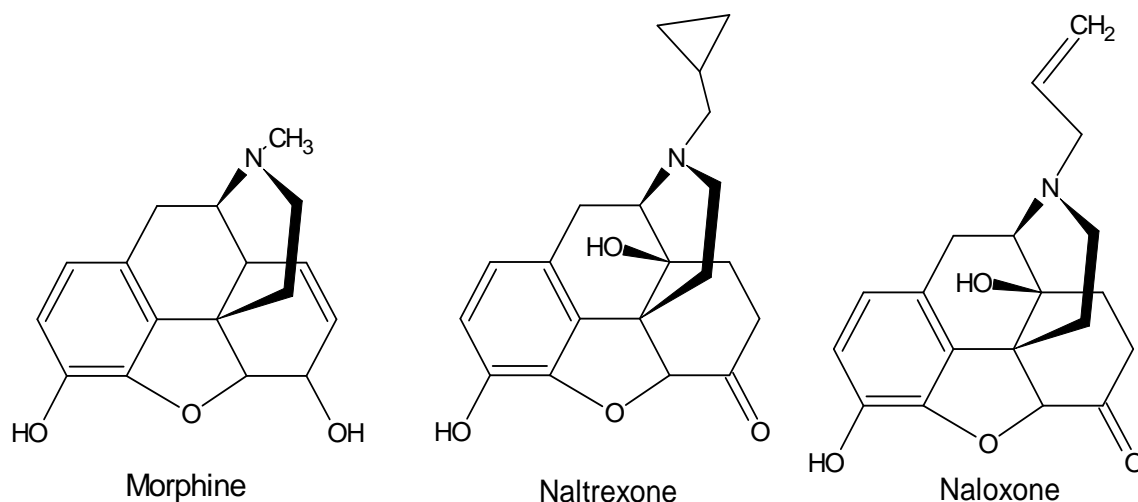


Figure 1.1. Chemical structures of morphine, naltrexone and naloxone (note degree of substitution on the N in the antagonists [naltrexone and naloxone] compared to morphine).

### 1.2.3. Opioid Receptors

#### 1.2.3.1. Discovery

It is beyond the scope of this thesis to fully describe the discovery and characterisation of opioid receptors and the reader is referred to the excellent reviews (Dhawan et al., 1996), (Pasternak, 1993), Law et al., (2000). A brief summary of opioid receptor pharmacology is outlined below.

It was long thought that there must be a substrate for the action of opioid-like drugs in the central nervous system (CNS), and that there were endogenous compounds that had the same effect as the exogenous agonists. The suggestion in 1954 that there were pharmacologically relevant opioid receptors based on the differential activities of chemical stereoisomers, was followed by the proposal in 1971 that radiolabelled compounds might be used to demonstrate the existence of these receptors and to characterise them. This was shown to be the case by independent research groups (Pert et al, 1973), (Terenius, 1973), when radioligands of high specific activity became available.

It was proposed by Martin and co-workers in 1976, that there were three different types of opioid receptor, and that these be named after the main ligands used to demonstrate activity at these receptors, i.e. mu ( $\mu$ ) for morphine, kappa ( $\kappa$ ) for ketocyclazocine and sigma ( $\sigma$ ) for SKF-10047 (Martin et al., 1976). Just prior to this however, the first endogenous peptide ligands for opioid receptors (the enkephalins and  $\beta$ -endorphin) were isolated (Hughes et al., 1975; Kosterlitz and Waterfield, 1975). Another group of peptides, the first of which was

named dynorphin was identified in the early 1980's by Goldstein and colleagues (Goldstein et al., 1981). These peptides were also used to classify the opioid receptors, both by radioligand binding studies to determine their location and densities within the CNS, and using classical bioassays with guinea pig ileum, and *vas deferens* of the mouse, rat, rabbit and hamster to study the properties of peripheral opioid receptors.

The *vas deferens* bioassay studies of Kosterlitz and co-workers resulted in the discovery of a fourth type of opioid receptor, the delta ( $\delta$ ), after *deferens* (Lord et al., 1977). Subsequently the sigma receptor has been shown to be non-opioid in nature (Dhawan et al., 1996). More recently, an opioid-like receptor has been cloned in humans and animals, bearing approximately 60% homology to the classical opioid receptors. In addition, the endogenous ligand for this receptor was isolated independently by two groups and named nociceptin and orphinan FQ, respectively, by the groups. The receptor itself has been named opioid-receptor like (ORL<sub>1</sub>), or the nociceptin opioid receptor (NOR), (Mogil and Pasternak, 2001).

Although there is some evidence for additional opioid receptor types, e.g. epsilon, zeta and lambda, these are not well-defined (Dhawan et al., 1996). The three main opioid receptor types are described in more detail below.

#### **1.2.3.2. Nomenclature**

The International Union of Pharmacologists (IUPHAR)-Nomenclature Committee recommended in 1996 that the names of the opioid receptors be rationalised on receptor type and order of demonstration of cloning and sequencing, and that these names be adopted by both pharmacologists and molecular biologists. Therefore, they recommended that all opioid receptors be labelled OP, and of the three well-defined receptors,  $\delta$  (the first cloned) would be OP<sub>1</sub>, with  $\kappa$  and  $\mu$  being named OP<sub>2</sub> and OP<sub>3</sub> respectively. Any new receptor identified would be OP<sub>4</sub>, OP<sub>5</sub> etc, and subtypes would be given an Arabic letter subscript after the numeral, e.g., 2  $\mu$ -receptor subtypes would be OP<sub>3A</sub> and OP<sub>3B</sub> (Dhawan et al., 1996). It has been suggested that the nociceptin/orphinan FQ receptor should be referred to as OP<sub>4</sub> (Calo et al., 2000).

Subsequent to this, the Committee revised this proposal, that the opioid receptors retain the OP for opioid peptide receptors, and their original Greek lettering in the capital form and be known as DOP, KOP and MOP, for  $\delta$ ,  $\kappa$  and  $\mu$  opioid receptors, respectively. They have also been referred to as DOR, KOR and MOR for  $\delta$ ,  $\kappa$  and  $\mu$  opioid receptors, respectively

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(Pasternak, 2001). The IUPHAR Database (2009) lists the receptors as  $\mu$ ,  $\delta$ , and  $\kappa$ , and the human gene names as *OPRM1*, *OPRD1* and *OPRK1*, for mu, delta and kappa, respectively, so that nomenclature is used in this thesis (Cox et al., 2008).

### **1.2.3.3. Structure and Function**

In the early 1990s, the three opioid receptor types were cloned, and from the sequence analysis of the cloned receptors, it has been shown that they belong to the guanine nucleotide binding (G)-protein coupled (GPCR) superfamily. This is the largest superfamily of receptors in the human genome, with >800 GPCR identified to date (Goddard and Abrol, 2007). In particular, opioid receptors appear to interact preferentially, but not exclusively with the pertussis toxin (PTX)-sensitive G-proteins of the  $G_i$  and  $G_o$  families. GPCR contain seven hydrophobic transmembrane domains that are linked by hydrophilic groups. These transmembrane domains are highly homologous among the three receptor types and intracellular protein kinase A and C consensus sites are conserved among the three receptors. The most divergent areas between the receptor subtypes are in the extracellular N terminus, the intracellular C terminus, and the long third intracellular loop (Law et al., 2000). The GPCR consist of heterotrimeric G proteins which consist of multiple isoforms of distinct  $G\alpha$ ,  $\beta$  and  $\gamma$  subunits, which mediate the actions of a wide variety of cell surface receptors. The receptors catalyse exchange of tightly bound guanine diphosphate (GDP) for guanine triphosphate (GTP) on the  $\alpha$  subunit, and this process requires the complete heterotrimer. The  $G\alpha$  subunit which is tightly bound to GDP interacts with the  $G\beta$  subunit in the inactive resting state. This is thought to help change the conformation of the  $G\alpha$  subunit and expose receptor binding sites. Binding of GTP results in activation of the G protein and dissociation of the  $G\alpha$  subunit from the  $G\beta\gamma$  subunits (Smrcka, 2008).

For opioids, GPCR activation is primarily by exhibiting inhibitory modulation of synaptic transmission in both the CNS and the myenteric plexus. Both mu and delta receptors mediate the inhibition of adenylyl cyclase (therefore inhibiting cyclic 3',5'-adenosine monophosphate [cAMP] formation (Raynor et al., 1995), and the activation of inwardly rectifying potassium channels (Mestek et al., 1995). Presynaptically, activation of mu receptors inhibits the opening of voltage-dependent calcium channels (N-type or L-type), whereas at the postsynaptic level, mu receptor activation opens potassium channels and hyperpolarizes neurons (Powell et al., 2002). The result of these changes is a decrease of both neuronal excitability and neurotransmitter release (Margas et al., 2007).

Studies have shown that GPCR form homo- and heterodimers. In the case of heterodimers, the receptors need to be co-expressed in the same cell. The Substance P receptor (neurokinin 1, NK1) and  $\mu$ -opioid receptors (MOR1) have been shown to form heterodimers (NK1-MOR1) in human embryonic kidney (HEK 293) cells, which leads to cross-phosphorylation and changes in the receptor internalization and desensitization (Pfeiffer et al., 2003).

While *in vitro* and *in vivo* studies suggest subtypes of mu, delta and kappa receptors exist, ( $\mu$ 1- $\mu$ 3,  $\delta$ 1,  $\delta$ 2,  $\kappa$ 1- $\kappa$ 3) molecular cloning and mouse knockout studies have really only supported the existence of one gene for each receptor type (Law et al., 2000; Dhawan et al., 1996). The three cloned opioid receptor genes are distributed on different chromosomes (chromosome 1 for delta, chromosome 6 for mu and chromosome 8 for kappa), all have multiple exons and span large distances in the chromosomal DNA (Law, 2000). All have multiple splice junctions, and have been shown to have splice variants that exhibit different pharmacological activities (Pan et al., 1999).

Additionally, for all three receptor types, mice have been generated that have the relevant receptor “knocked out”. These receptor knockout animals exhibit specific reduction in the receptor protein levels without changing the other receptor types (Law et al., 2000).

All three receptor types have been shown to exhibit basal (constitutive) signalling activity in cell culture assays and in mouse brain tissue in the absence of agonists (Liu et al., 2001; Wang et al., 2007; Sadee et al., 2005). In the case of  $\mu$ -opioid receptors, this leads to the regulation of intracellular effectors including the inhibition of adenylyl cyclase activity, the closing of voltage-gated  $\text{Ca}^{2+}$  channels and the activation of inwardly rectifying  $\text{K}^{+}$  channels (Liu et al., 2001), all effects seen in the presence of an agonist. Ligands that can reduce this spontaneous, agonist-independent activity (and in most cases block the actions of an agonist) are termed inverse agonists, while ligands that block the action of agonists without affecting basal signalling activity are termed neutral antagonists (Liu and Hong, 2003), (Raehal et al., 2005), (Sadee et al., 2005). It is thought that better understanding of the mechanisms underlying the effect of ligands on the basal activity of receptors may lead to advances in prediction of therapeutic response, and insights into the mechanisms of the development of tolerance and dependence. The pharmacological actions of naltrexone and 6 $\beta$ -naltrexol on basal signalling activity of  $\mu$ -receptors in particular, are discussed below.

#### **1.2.3.4. Delta ( $\delta$ ) Receptors**

The delta receptor has the enkephalins as the highest affinity endogenous ligands. The precursor for enkephalins is the polypeptide proenkephalin. With few exceptions, all endogenous delta receptor agonists are peptides derived from the enkephalins. Two of these are  $\Delta$ -Ala- $\Delta$ -leu-enkephalin, (DADLE), and the hexapeptide DSLET, which has 20-600-fold selectivity for delta over mu or kappa receptors. The naltrexone derivative naltrindole (NTI) was the first selective and potent delta receptor antagonist to be synthesised, however it is an agonist at kappa receptors. A slightly less potent, but more selective antagonist at delta receptors is TIPP, a tetraisoquinoline-substituted, deltorphin-related tetrapeptide. The distribution of delta receptors in the CNS is more restricted than the other receptor types. The highest receptor densities, as measured by the positron emission tracer N1'-([<sup>11</sup>C]methyl)naltrindol were found in neocortex (insular, parietal, frontal, cingulate and occipital), caudate nucleus and putamen, whilst binding was intermediate in the amygdala and lowest in the cerebellum (Madar et al., 1996). The main function of delta receptors is analgesia, considered to be mediated by an inhibition of substance P release from terminals of nociceptive primary afferent fibres. Delta receptors appear particularly effective toward thermal and chemical stimuli. Both peripheral and central delta receptors seem to be involved in the inhibition of gastrointestinal transit by selective agonists, and medullary delta receptors are important for cardiovascular regulation, possibly participating in the effects of the  $\alpha_2$ -agonist clonidine, by interactions between the pre-synaptic alpha-2B receptors and delta receptors (Dhawan et al., 1996), (Gyires et al., 2001). None of the opioid compounds used therapeutically have actions exclusively at the delta receptors.

#### **1.2.3.5. Kappa ( $\kappa$ ) Receptors**

The endogenous ligands of kappa receptors are the dynorphins, from the precursor prodynorphin, which have high affinity, but limited selectivity for these receptors. The morphine derivative nor-binaltorphimine is a potent antagonist with an unusually long duration of action. There is marked species variation with regard to the distribution of these receptors, with guinea pigs having the greatest density in the inner layers of the cerebral cortex. By contrast, the rat has only low levels of radioligand binding throughout the cerebral cortex, with the highest densities being observed in the nucleus accumbens, claustrum, dorsal endopiriform nucleus and interpeduncular nucleus (Dhawan et al., 1996). In the human forebrain, high densities of kappa sites are present in the deep layers (laminae V and VI) of the cortex, claustrum, nucleus basalis of Meynert, and amygdaloid body. Lower densities are observed in the caudate, putamen, globus pallidus and nucleus accumbens (Quirion et al.,

1987).

The functions of the kappa receptors include nociception, diuresis, hunger recognition and neuroendocrine secretions. Both central and peripheral kappa receptors mediate the anti-diarrhoeal properties of opioids. Kappa receptors could also be involved in thermoregulation and modulation of cardiorespiratory function in the rat (Dhawan et al., 1996). Kappa receptor agonists are not reinforcing in non-human species, and can produce dysphoria in humans (Dhawan et al., 1996).

#### **1.2.3.6. Mu ( $\mu$ ) Receptors**

In the context of this thesis, the  $\mu$ -opioid receptor is the most important of the opioid receptors as naltrexone and 6 $\beta$ -naltrexol have higher affinity for this receptor than either  $\delta$  or  $\kappa$  opioid receptors as described below. It is also the  $\mu$  opioid receptor that is the most important in analgesia, and the development of tolerance and dependence with chronic use of opioids (Matthes, 1996). This has been shown in C57BL/6 mice with the  $\mu$ -opioid receptor gene 'knocked out'. In these animals morphine analgesia was abolished in the tail-flick (spinal) and hotplate (supraspinal) tests, and there was no morphine-induced dependence as shown by a complete lack of withdrawal signs in chronically-treated animals (Matthes et al., 1996).

The probable endogenous ligands for the mu opioid receptor are the endomorphins 1 and 2. Both have been shown to have a very high affinity ( $K_i = 360$  and  $690$  pM) and selectivity (4000-15000-fold, and 8000-14000-fold preference for  $\mu$  over  $\kappa$  and  $\delta$  receptors, respectively), for endomorphins 1 and 2, respectively, in the mouse brain (Zadina et al., 1997).  $\beta$ -endorphin, from pro-opiomelanocortin binds to the mu and delta receptors with equal affinity (Zadina et al., 1997). Morphine, the best known exogenous opioid, has a higher affinity for mu receptors than either of the other receptor types. Two antagonists which have higher affinity for mu receptors than delta or kappa are naloxone and naltrexone (Gianoulakis et al., 1983).  $\beta$ -funaltrexamine acts as an irreversible mu receptor antagonist, but also as a reversible kappa receptor agonist (Ward et al., 1985).

As shown by autoradiographical studies with selective radioligands, mu receptors are distributed throughout the neuroaxis. The highest density of these receptors is in the caudate putamen, but they are also found in the neocortex (laminae I and IV), thalamus, nucleus accumbens, hippocampus and amygdala (Pilapil et al., 1987). Mu receptors are also present in the superficial layers of the dorsal horn of the spinal cord, where they are located in part on

the presynaptic terminals of nociceptive primary afferent fibres (Dhawan et al., 1996).

While it has been postulated that subtypes of the mu opioid receptor based on binding and pharmacological studies, only one receptor has been cloned (Mestek et al., 1995; Wang et al., 1993). Since the cloning of the  $\mu$ -opioid receptor gene (Wang et al., 1993), (Raynor et al., 1995) there have been found to be a number of splice variants of the receptor (Pasternak, 2001; Pan et al., 1999; Pan et al., 2000). At least 10 variant forms have been identified, (designated MOR-1A to MOR-1X), some of which express truncated forms of the receptor, or variations in the intracellular tip of the C-terminus of the receptor (Cox et al., 2008; Pasternak, 2005). These have been defined using knockout mice, antisense mapping studies and studies showing subtype differences in agonist affinity and analgesia.

Studies have shown that heroin and morphine-6-glucuronide (M6G) have a high affinity for a mu receptor subtype distinct from that mediating the effects of morphine (Brown et al., 1997b). The opioid antagonist 3-methoxynaltrexone was shown by these authors to selectively antagonise the antinociceptive actions of heroin and M6G, without affecting the antinociception of mice *in vivo* by morphine, and selective delta and kappa agonists (Brown et al., 1997a). In a recent study it was shown that exon 11 knockout mice had a normal response to morphine and methadone in the tail-flick assay, but a decreased response to heroin, M6G and fentanyl. In the hotplate assay, there was no effect on morphine, but a significant decrease in the analgesic actions of M6G in the exon 11 knockout mice. Naloxone (5 mg/kg sc) eliminated the analgesic response of both drugs in wild-type and knockout mice. The analgesic phenotype observed in this study is different from that seen with exon 1 knockout mice, and splice variants involving exon 11 may offer insights into the different actions of heroin and M6G compared to morphine (Pan et al., 2009).

In addition to splice variants of the gene, single nucleotide polymorphisms (SNP) have been observed, which can affect protein expression and receptor binding, and ultimately play a role in predisposition to tolerance and dependence, and response to naltrexone therapy (Zhang et al., 2005). One particular SNP polymorphism which has been found in high abundance in Caucasian (~15%) and especially Asian populations (~40%) occurs at position 118 (A118G) in exon 1, and results in an amino acid change from asparagine to aspartate at position 40 of the receptor (Asn<sup>40</sup>->Asp) (Margas et al., 2007). Another exon 1 polymorphism results in an amino acid change at position 17 from alanine (C) to valine (T), (C17T) at position 6 of the receptor. The Val6 allele is found predominantly in people of African descent (Oslin et al., Susan J Porter, PhD Thesis, 2010

2003). Additionally, in the human receptor gene, a rare Ser<sup>268</sup>->Pro polymorphism has also been identified, and shown to possess a marked reduction in coupling efficiency and is less desensitised upon agonist exposure (Cox et al., 2008).

The A118G polymorphism has been reported to have associations with the perception of pain in various ethnic populations. Tan and colleagues conducted a study in three main Asian ethnic groups (Chinese, Malay and Indian), on the association of A118G on self-reported pain and self-administered morphine analgesia after surgery (lower segment caesarean section). They found that the 118G variant was associated with higher pain scores, higher morphine usage and lower nausea scores. With regard to ethnicity, the group with the lowest frequency of 118G (Chinese), also had the lowest morphine consumption and pain scores (Tan et al., 2009).

In a preliminary study on alcohol-dependent people, these two polymorphisms were examined for association between drinking outcomes in patients treated with naltrexone or placebo. As the authors report, there were limitations in this study regarding sample size and the fact that the genetic vulnerability of patients was not the main focus of the larger study from which the sample population came. However, they found that there was a significantly greater proportion of naltrexone-treated (100 mg/day) European American subjects with the Asp40 variant (A/G or G/G) who did not return to heavy drinking compared to those homozygous for the Asn allele (A/A). Unfortunately, the African American sample size was too small to show any differences in the C17T polymorphism (Oslin et al., 2003).

A later study using a much larger population (within the Combined Pharmacotherapies and Behavioral Interventions for Alcohol Dependence [COMBINE]) study, also examined the effect of the presence of the Asp40 variant on naltrexone treatment-response in alcohol-dependent subjects (Anton et al., 2008). The outcome measures in this study were time trends in percentage days of abstinence, percentage of heavy drinking days and rates of good clinical outcome (defined in the COMBINE trial as abstinent or moderate drinking without problems, specified maximum numbers of drinks and drinking days, and reduction in alcohol-related drinking problems). The authors reported that alcoholic patients with an Asp40 allele receiving medical management alone (without combined behavioral intervention) had better responses with naltrexone compared to placebo in days abstinent, and number of drinking days. Patients with the wild-type Asn40 allele did not show medication differences. Those patients carrying the Asp40 allele also had a higher percentage of good clinical outcome with

naltrexone treatment compared to placebo-treated patients with Asp40 or Asn40 and naltrexone-treated patients with Asn40. The authors conclude that the Asp40 allele helps predict naltrexone-treatment response, and that genotyping patients prior to treatment may be useful in the design of treatment options (Anton et al., 2008).

As mentioned above,  $\mu$ -opioid receptors display basal signalling. This activity results in G-protein stimulation and adenylyl cyclase inhibition, which leads to upregulation of the cAMP second messenger system. This constitutive activity is enhanced with chronic exposure to opioids in membrane preparations (Liu et al., 2003; Wang et al., 1994). Basal signalling in the  $\mu$ -opioid receptor was established in tissue culture cells usually transfected with the receptor. Ligands that can reduce this spontaneous, agonist-independent activity (and in most cases block the actions of an agonist) are termed inverse agonists, while ligands that block the action of agonists without affecting basal signalling activity are termed neutral antagonists (Liu et al., 2003; Raehal et al., 2005; Sadee et al., 2005). Methods using  $^{35}\text{S}$ -GTP $\gamma$ S binding in mouse brain tissues showed that basal  $\mu$ -opioid receptor activity accounted for nearly 40% of maximal stimulation in the dependent state (Sadee et al., 2005). Previously, these researchers had found that naloxone acted as a neutral antagonist (no effect on basal activity) in the morphine-naïve state, and an inverse agonist (blocks basal activity) in the morphine-dependent state. These researchers also showed naltrexone to be an inverse agonist, and 6 $\beta$ -naltrexol to be a neutral antagonist (Wang et al., 2001). Naltrexone and naloxone are said to be 'protean' antagonists due to their ability to change from neutral antagonists to inverse agonists depending on the nature of the receptor under all conditions studied (Wang et al., 2007).

#### **1.2.4. Receptor Tolerance and Dependence**

Continued stimulation of opioid receptors by either agonists or antagonists results in adaptational changes in receptor and second messenger function. These changes are thought to contribute to the development of tolerance and physical dependence. Tolerance is characterised by a decrease in the effect of the drug following repeated administration which can be overcome by increasing doses of the drug. Physical dependence is manifested as a withdrawal syndrome following cessation of the drug. Chronic treatment with morphine leads to tolerance to its analgesic and respiratory actions, but not pupillary constriction and constipation, whereas chronic treatment with naltrexone or other opioid antagonists can lead to receptor up-regulation and super-sensitivity to agonists such as morphine. This up-regulation has been reported to be due to an increased number of receptors and not a change

in the affinity of the opioid receptors, with all three classes of receptor being affected by long-term naltrexone use. These changes have been reported to be time-, receptor-type, and site-dependent (Mattick et al., 1998).

The receptor down-regulation with chronic agonist treatment varies in different studies and may depend on the brain region studied. It is now thought that intracellular changes are probably more important in mediating tolerance and dependence, such as regulation of phosphorylation of receptors (Nestler, 1997). At the post-receptor signal transduction level, chronic exposure to opioid agonists leads to substantial increases in adenylyl cyclase activity possibly due to changes in levels of specific G-protein subunits, and in the individual proteins that comprise the cyclic AMP system in a number of opiate-responsive neurons in the central nervous system. This has been shown to occur in the locus coeruleus, nucleus accumbens and dorsal root ganglion neurons (Nestler and Aghajanian, 1997). In cell culture studies, tolerance is associated with receptor desensitization/uncoupling, internalization, and degradation pathways. Desensitization was shown to involve receptor phosphorylation in the carboxyl tail and/or third intracellular loop (i3). In contrast to the contribution of desensitization to tolerance, dependence is thought to involve up-regulation of the cAMP second messenger system. The association of calmodulin (CaM), and subsequent dissociation of CaM from the receptor and plasma membrane following agonist treatment have been shown in HEK 293 cells to increase basal  $\mu$ -opioid receptor coupling to G-protein, after prolonged treatment with morphine,  $\mu$ -opioid receptor to G protein was reduced, and desensitization was apparent, indicating that removal of CaM may play a role for the development of tolerance, but is not the major mechanism (Wang et al., 2000).

Studies have also shown that continued stimulation of opioid receptors by agonists causes excitatory signalling, possibly by the receptor coupling to  $G_s$  rather than  $G_{i/o}$  proteins in this condition. Tolerance is considered to be caused by a sensitization to this coupling which leads to activation of adenylyl cyclase, elevation of cAMP and activation of sensory neurotransmitters. This induces a hyperalgesia which antagonizes the analgesic effects of the  $G_{i/o}$ -coupled analgesic response (Crain and Shen, 1998).

Since the cloning of the human  $\mu$  opioid receptor, a number of studies have examined possible mechanisms of tolerance at the molecular level. In one study, exposure of cells expressing human and rat  $\mu$  receptors to 1 mM morphine showed no change in binding of radiolabeled agonists (Raynor et al., 1995). In a separate study, using oocytes expressing the



human  $\mu$  receptor and the inwardly rectifying  $K^+$  channel, sequential activation of the receptor with [D-Ala<sup>2</sup>,N-MePhe<sup>4</sup>,Gly-ol<sup>5</sup>]enkephalin (DAMGO- a selective  $\mu$  opioid receptor agonist) resulted in a desensitization of the  $K^+$  channel. This desensitization was potentiated by protein Kinase C (PKC) and calmodulin (CaM) kinase (Mestek et al., 1995).

### **1.3. Naltrexone and 6β-Naltrexol**

#### **1.3.1. Naltrexone History**

Naltrexone was synthesised in 1963 by Matossian (Blumberg and Dayton, 1974). The development of naltrexone was initiated by the need for a potent, long-lasting orally active narcotic antagonist, for the treatment of opioid overdose and dependence.

#### **1.3.2. Chemistry of Naltrexone and its Metabolites**

Naltrexone, 17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxymorphinan-6-one, has a molecular weight of 341.4, a chemical composition of C<sub>20</sub>H<sub>23</sub>NO<sub>4</sub>, is a cyclopropyl derivative of the phenanthrene opioid oxymorphone and is structurally similar to naloxone and morphine (Figure 1.1). Naltrexone is a white crystalline powder with a melting point of 168-170 °C. It is usually synthesised and used as the hydrochloride salt (MW 377.9), which has a melting point of 274-276 °C, and is highly soluble in water. Naltrexone has two pK<sub>a</sub> values, one for the proton on the nitrogen, and one for the phenolic proton. The pK<sub>a</sub> values for the hydrochloride salt of naltrexone have been reported as 8.38 at 20 °C and 8.3 at 37 °C for the proton on the nitrogen, and 9.93 and 9.51 at 20 and 37 °C respectively, for the phenolic proton (Kaufman et al., 1975a). The lower pK<sub>a</sub> value was used to determine the octanol:water ratio, which for naltrexone hydrochloride was 45.6 at 20 °C and 83.33 at 37 °C. These values were much lower than those reported for naloxone hydrochloride showing that although similar in structure, naltrexone is significantly less lipophilic than naloxone. This could theoretically result in a slower onset of action, and longer duration of action of naltrexone compared to naloxone. The drug distribution coefficient was shown to be pH-dependent, increasing with increasing pH, from 2.3 at pH 7.1 to 7.9 at pH 7.7 at 20 °C. The temperature dependence was more marked, with the octanol:water coefficient being 4.32 at 20 °C and 13.1 at 37 °C, at the physiological pH of 7.40 (Kaufman et al., 1975b).

The distribution coefficient (D) mentioned above describes the ratio of the sum of the concentrations of all forms of the compound (ionized plus unionized). Lipophilicity of a compound is often described in terms of its partition coefficient *P* (or log *P* as it is usually expressed), which describes the intrinsic lipophilicity of the compound in the absence of

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dissociation or ionization (Manners *et al.*, 1988).

While there is a huge database of compounds which have had partition coefficients measured using octanol:water, Wang and Lien (1980) showed that for basic drugs (such as naltrexone), there is not a 1:1 correlation between octanol:water and octanol:buffer when measuring the partition coefficients at pH 7.40 (Wang and Lien, 1980). Therefore, it is important to determine the partition coefficient of naltrexone using octanol:pH 7.4 phosphate buffer.

6 $\beta$ -naltrexol (N-cyclopropylmethyl-4,5-epoxymorphinan-3,6,14-triol) is a white crystalline powder with a molecular weight of 343. The free base is practically insoluble in water, but readily soluble in methanol.

At the time this PhD study commenced in 1997, 6 $\beta$ -naltrexol was not commercially available, and to obtain pure compound, most of the early studies used 6 $\beta$ -naltrexol extracted from urine of volunteers taking naltrexone, as their standard. The alternative was a chemical reduction of naltrexone (Malspeis *et al.*, 1975). These researchers used lithium tri-sec-butylborohydride to reduce naltrexone, but this resulted in the production of 6 $\alpha$ -hydroxy naltrexol only. A later method involved a stereospecific reduction of the carbonyl group at carbon 6, using formamidinesulfinic acid (thiourea dioxide) under nitrogen in an aqueous, alkaline medium at 85°C. This reduction yielded 88.5% product, of approximately 97% the 6 $\beta$ -hydroxy epimer which is more acceptable for use as a standard (Chatterjee *et al.*, 1975). The structures of the alpha and beta reduced products of naltrexone are shown below.

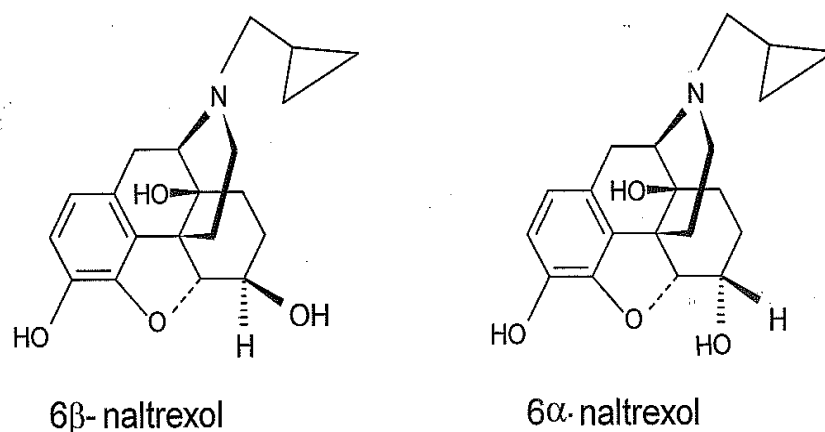


Figure 1.2. Comparative structures of 6 $\beta$ -and 6 $\alpha$ -naltrexol  
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Other methods of synthetic reduction of naltrexone have been used, and although the yields were high (98%), they usually gave mixtures of products. One such method used sodium borohydride as the reducing agent, and a chloroform extraction, but this resulted in a mixture of 6 $\alpha$ -:6 $\beta$ - naltrexol of 85:15 (Malspeis et al., 1975). Ahmed and colleagues (1979) also used sodium borohydride (2 molar equivalents) to reduce naltrexone, but they did not report either yield or purity (Ahmed et al., 1979).

#### 1.4. Determination of Naltrexone and its Metabolites in Biological Fluids

There have been a number of published methods for the quantification of naltrexone and/or 6 $\beta$ -naltrexol in biological fluids. Many of these have involved substantial sample work-up, consisting of complicated extraction and derivatisation procedures, the use of radiolabelled compounds and specialised equipment such as GC/MS, GC/MS/MS and liquid scintillation counters. Davidson and colleagues in 1996 published a method for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in human plasma and urine, using minimal extraction procedures and HPLC with electrochemical detection (Davidson et al., 1996). The method presented in the current thesis is based on that by Davidson *et al* (1996). A summary of previously used methods for the determination of naltrexone and 6 $\beta$ -naltrexol is outlined below.

Saliva is a non-invasive, easily collectable biological fluid and as such could become increasingly useful in the detection and quantification of many compounds. Few researchers have examined the concentrations of naltrexone or 6 $\beta$ -naltrexone in saliva following administration of naltrexone. Verebey *et al* (1980) examined the differential distribution of naltrexone, 6 $\beta$ -naltrexol and 2-hydroxy-3-methoxy 6 $\beta$ -naltrexol (HMN) in plasma, red blood cells, saliva and urine (method described in 1.4.2 below). The authors concluded that measuring 6 $\beta$ -naltrexol or HMN in plasma, saliva or urine, could more efficiently monitor naltrexone status than measuring naltrexone itself in any body fluids. Based on the data presented (Table II excluding Subject 3, 16 hr sample), the mean ( $\pm$  SD) saliva:plasma concentration ratios were 1.36 (0.47) for naltrexone, and 0.81 (0.29) for 6 $\beta$ -naltrexol, respectively.

6 $\beta$ -Naltrexol formation has been assessed in human liver cytosol by measuring the oxidation rate of NADPH at 340 nm, with identification of products by thin layer chromatography

(TLC) (Ohara et al., 1995), but prior to the current study, this metabolite had not been quantified in this tissue.

#### **1.4.1. Thin layer chromatography (TLC)**

Misra and colleagues examined the disposition of radiolabelled [15,16-<sup>3</sup>H]naltrexone in the central nervous system and blood of the rat following injection into the dorsal area above the right hind limb (Misra et al., 1976). The qualitative detection of naltrexone and its metabolites in plasma and brain homogenate was performed using TLC. After solid-phase extraction of acid hydrolysed samples, methanol eluants were separated on Gelman ITLC (silica gel) sheets with various solvent systems. The best solvent for the detection of naltrexone and 6 $\alpha$ - and 6 $\beta$ -naltrexol comprised *n*-hexane/ethyl acetate/concentrated NH<sub>4</sub>OH (60:40:0.1 v/v), while the more polar metabolites were detected using ethyl acetate/methanol/concentrated NH<sub>4</sub>OH (17:2:1 v/v). The R<sub>f</sub> values obtained using this system for naltrexone and 6 $\beta$ -naltrexol were 0.9 and 0.44, respectively. The use of different solvent systems resulted in good selectivity for naltrexone and its metabolites.

Ludden and colleagues also subjected urine samples from rats and guinea pigs administered [15,16-<sup>3</sup>H]naltrexone to TLC analysis (Ludden et al., 1978). After considerable work-up, the extract residues were spotted onto silica gel on alumina using solvent systems of A) chloroform/methanol/ concentrated NH<sub>4</sub>OH 46 ml:4 ml:2 drops v/v) or B) methanol/*n*-butanol/benzene/water (60:15:10:5 v/v). The R<sub>f</sub> values obtained for naltrexone and 6 $\beta$ -naltrexol were A) 0.55 and 0.16, and B) 0.34 and 0.32, respectively. The TLC products were scraped from the plate and the radioactivity measured. The results were expressed as a percentage of the administered naltrexone dose. In the 48 hour period after a 1mg/kg dose of naltrexone 1.4% of the dose was recovered in urine as free and conjugated 6 $\alpha$ -naltrexol, 11.4% as free and conjugated 6 $\beta$ -naltrexol and 58% as the parent compound, of which 57% was as the conjugate.

Solvent system B was not useful in separating naltrexone from its reduction products, and solvent system A was used to quantify the excretion products as there was good separation of compounds.

#### **1.4.2. Gas chromatography (GC)**

Urine and plasma samples from rhesus monkeys were analysed by electron capture GC to determine the disposition and pharmacokinetics of naltrexone after IV and oral administration  
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(Reuning et al., 1989). The samples were extracted with either benzene (extraction efficiency >70%) or ethyl acetate (extraction efficiency >85%) and derivatised with pentafluoropropionic anhydride (PFPA). The derivatives were chromatographed on a GC equipped with a <sup>63</sup>Ni-electron-capture detector. The coiled glass column packed with 3% OV-17 on 100-120 mesh support, had an internal diameter of 2 mm and was either 1.8 m or 2.4 m in length. The oven temperature was 203 or 205°C, with carrier gas (argon 95:methane 5) flow rates of 40 or 24-30 ml/min, depending on the column used. Confirmation of the correct chromatography peaks was performed by comparison of pure derivatised samples. The sensitivity (lower limit of quantification) of the assay was 0.3 ng/ml.

In humans, Verebey *et al* (1980) used GC to quantify naltrexone, 6β-naltrexol and 2-hydroxy-3-methoxy-6β-naltrexol (HMN) in plasma, red blood cells, saliva and urine. This method involved extraction with chloroform/benzene or benzene alone, and derivatisation with bistrimethylsilyltrifluoroacetamide (BSTFA) or PFPA. The column was a 2 m x 2 mm glass spiral packed with 3% OV-17 on Gas-Chrome 80-100 mesh. Temperatures of the detector and flash heater were 285°C for urine analyses, and 300 and 240°C for the assay of other body fluids, with a column oven temperature of 230°C for the urine assay and 220°C for the assay of other fluids. The limits of detection were 10-20 ng/ml for urine and 0.5 to 1.0 ng/ml for the other body fluids. Due to the inability to separate PFPA-derivatised HMN and 6β-naltrexol with this system, 6β-naltrexol was determined by subtracting the HMN values obtained with benzene extraction from the values obtained for both metabolites from the chloroform-extracted samples. The assay specificity and the use of quality control samples to monitor the ongoing performance of the assay were not reported.

#### **1.4.3. Mass spectrometry (LC-MS[MS], GC-MS[MS])**

Wall and colleagues (1981) studied the metabolism and disposition of radiolabelled naltrexone in humans after oral and intravenous administration. They used a combination of liquid scintillation spectrometry, thin layer chromatography, <sup>13</sup>C NMR spectrometry and GC/MS to quantitate the concentrations of naltrexone, 6β-naltrexol and HMN (2-hydroxy-3-methoxynaltrexol) in plasma and urine. The GC/MS analysis of urine samples involved acid and base extractions into chloroform, and derivatisation with methoxyamine hydrochloride in pyridine and Tri-Sil TBT. The chromatography column used was 1.8 mm x 4 mm i.d. packed with 2% OV-17 on 100/120 mesh. The column temperature was 230°C, and the mass spectrometer was operated with an ionising voltage set at 70eV. The limits of detection and precision and accuracy data were not reported.

An LC-MS assay was developed to assay naltrexone and 6 $\beta$ -naltrexol in the smaller volumes obtainable in animal studies (Valiveti et al., 2004). This assay required only 100  $\mu$ l of plasma, and a simple protein precipitation and extraction method. The limit of quantification for both analytes was 1.25 ng/ml, with an accuracy of > 95% with inter-assay precision of  $\leq$  7%. The chromatography was performed on a Waters Symmetry C<sub>18</sub> column (2.1 mm x 150 mm x 5  $\mu$ m) with a Waters Symmetry C<sub>18</sub> guard column (2.1 mm x 10 mm). The LC-MS system consisted of a Waters Alliance 2690 HPLC pump, A Waters Alliance 2690 autosampler, and a Micromass ZQ detector using electrospray ionization for ion production. Selected ion monitoring (SIM) was performed in positive mode for naltrexone, 6 $\beta$ -naltrexol and the internal standard naloxone. As SIM was used for detection, there were no interfering peaks observed in the analysis of blank plasma extracts (Valiveti et al., 2004).

A GS/MS method has been developed to measure naltrexone and 6 $\beta$ -naltrexol in human plasma following implantation of naltrexone pellets (Toennes et al., 2004). This method was linear up to 60 ng/ml for naltrexone, 200 ng/ml for 6 $\beta$ -naltrexol, with a limit of detection of 0.1 ng/ml. The extraction recoveries of 10 ng/ml of substrate were 83% for both analytes. Inter- and intra-day precision were <10% for naltrexone and <11% for 6 $\beta$ -naltrexol. The accuracy was in the range of 98-102% at all levels tested for both analytes.

Studies described below (1.6.1.8) examining slow-release preparations utilised LC-MS/MS. In one such study, subjects received either a single im injection of long-acting naltrexone (190 mg-n=12; 380 mg-n=12), or 4 x 380 mg every 28 days (n=12), and samples of 500  $\mu$ l of plasma from healthy volunteers were analysed. The assay had a limit of quantification of 0.2 ng/ml for naltrexone and 0.5 ng/ml for 6 $\beta$ -naltrexol. The accuracy ranged from 0.5 to 5.5% deviation from the theoretical concentrations for naltrexone and 6 $\beta$ -naltrexol, respectively, with CV < 12% for both analytes (Dunbar et al., 2006).

In an earlier study in alcohol-dependent subjects (n=16), naltrexone was administered as two 150 mg doses im on a single occasion (Galloway et al., 2005). Naltrexone and 6 $\beta$ -naltrexol in plasma (volume not reported), were analysed by LC-MS/MS. The assay had limits of quantification of 0.1 and 0.4 ng/ml, for naltrexone and 6 $\beta$ -naltrexol, respectively. The assay imprecision was <8% for both analytes and absolute deviation from nominal values < 7.5% for both analytes.

More recently, a highly sensitive LC-MS/MS has been developed with a lower limit of quantification of 5 pg/ml (Clavijo et al., 2008). In this assay, 100 µl of sample were protein precipitated in 96-well plates, then extracted online. The HPLC system was interfaced with the MS with an atmospheric pressure chemical ionization source. The MS was run in the positive multiple reaction monitoring mode with declustering potential set to 150 V, entrance potential of 10 V and collision energy of 35 eV with an interface of 300 °C. In addition to being very sensitive, the assay was linear from 0.005 to 100 ng/ml, and the interday accuracy and variability at 0.03 ng/ml were 103.7 % and 10.1% for naltrexone and 105.6% and 10.2% for 6β-naltrexol. While this is a very good assay, it requires sophisticated, expensive equipment.

#### **1.4.4. High Pressure Liquid Chromatography (HPLC)**

Further to the above-mentioned study, Wall and colleagues (1984) examined the disposition of naltrexone in man after subcutaneous administration of [15,16-<sup>3</sup>H<sub>2</sub>]naltrexone. In this study they used HPLC with UV detection. They used a Partisil 10/PAC column (0.46x 0.25 cm) in a 0.1% (w/v) aqueous ammonium acetate (pH 2.75)/*t*-butanol/acetonitrile (5:1:94) solvent system. The column effluents were monitored for UV absorbance at 254 nm and collected in fractions corresponding to the retention time (Rt) values of naltrexone and metabolites. These fractions were then quantified by liquid scintillation spectrometry. The coefficients of variation for naltrexone and 6β-naltrexol ranged from 3.5 to 14% for 0.1-20 ng/ml and 1.1 to 21% for 0.1-10 ng/ml, respectively, while %CV (coefficient of variation) values for the HMN metabolite were 4.4, 6.1 and 38% for 1.0, 0.5 and 0.1 ng/ml, respectively. While this assay was sensitive and specific, large sample volumes (3 ml) were used.

The effect of liver cirrhosis on the systemic availability of naltrexone in humans has been determined using reverse-phase HPLC analysis of human blood samples after solid-phase extraction (Bertolotti et al., 1997). The chromatography conditions were as follows: the HPLC was equipped with a diode array detector module set at 202 nm and a reversed-phase C-18 25cm x 4.6 mm Hypersil column packed with 5µm ODS. The mobile phase comprised acetonitrile (18%), 20 mM KH<sub>2</sub>PO<sub>4</sub> (82%) with 0.015% v/v triethylamine pH 3, with a flow rate of 1.0 ml/min. The intra- and inter-day precision coefficients of variation were 4.8% and 7.1%, respectively, for naltrexone and, 4.2% and 6.9%, respectively, for 6β-naltrexol, at drug concentrations of 10, 100 and 500 ng/ml. The sensitivity level for naltrexone and 6β-naltrexol was higher than that for earlier assays at 10 ng/ml for both drugs, which was sufficient in this study where the patients received a 100 mg bolus, but when smaller doses are administered, a

more sensitive assay is needed (Bertolotti et al., 1997).

In an earlier study by Meyer *et al* (1984), reversed-phase HPLC with ECD and an octyl column were used to determine the bioequivalence, dose-proportionality and pharmacokinetics of naltrexone after an oral dose to humans. Naltrexone and 6 $\beta$ -naltrexol were assayed simultaneously in plasma (2.0 ml) with limits of detection of 0.2 and 0.5 ng/ml, respectively for the two compounds. Intraday assay coefficients of variation (CV) were 22.5% (0.2 ng/ml) and 0.26% (100 ng/ml) for naltrexone and 11.8% (1 ng/ml) and 6.1% (100 ng/ml) for 6 $\beta$ -naltrexol. The corresponding interday coefficients of variation for the two compounds were 10.9% (0.5 ng/ml) and 0.46% (100 ng/ml) for naltrexone and 22.5% (3 ng/ml) and 0.51% (500 ng/ml) for 6 $\beta$ -naltrexol. While the extraction recoveries for naltrexone (96%) and the internal standard, an N-cyclopentylmethyl analogue of nalbuphine (88%) were high, that for 6 $\beta$ -naltrexol was only 45%. In the same study, urine concentrations of naltrexone and 6 $\beta$ -naltrexol had to be measured separately due to the high concentrations of 6 $\beta$ -naltrexol in the samples (Meyer et al., 1984). The intraday urine assay CV for naltrexone in urine ranged from 0.53-3.88% for 1-20  $\mu$ g/ml, while for 6 $\beta$ -naltrexol the range was 0.21% to 2.01% at 5-100  $\mu$ g/ml. The inter-assay CV values for naltrexone were 9.8% at 0.05  $\mu$ g/ml and 1.3% at 1  $\mu$ g/ml, and for 6 $\beta$ -naltrexol 5.6% (5  $\mu$ g/ml) and 0.63% (100  $\mu$ g/ml). The extraction recoveries were greater than 92% for naltrexone and 86% for 6 $\beta$ -naltrexol. While this assay is sensitive and precise, 2 ml of plasma were required for analysis.

#### **1.4.5. Summary**

As can be seen from the above, whilst there have been many methods developed for the quantification of naltrexone and 6 $\beta$ -naltrexol in biological fluids, many have been time-consuming and complicated, using specialised equipment and software not available at the time the studies reported in this thesis were conducted. While the sensitivity of most of the assays is good, often at least 2 ml of sample were required. The precision and accuracy were often not reported, so it is not possible to determine how well the assay performed over time.

When the studies reported in this thesis were conducted in 2000, GC and MS were not available to the author. Davidson had previously shown that HPLC with electrochemical detection (ECD) needed only a simple extraction and had good sensitivity (0.25 ng/ml). Accordingly, as described on page 59 of this thesis, that method was chosen for the studies reported below.



## **1.5. Metabolism of Naltrexone**

### **1.5.1.1. Introduction**

Most biotransformation of drugs involves two types of reactions, which often occur sequentially, and are known as Phase I and Phase II reactions. Phase I reactions usually consist of oxidation, reduction or hydrolysis, resulting in the formation of a reactive group on the molecule (such as an hydroxyl group). This functional group can then be conjugated (Phase II), forming a larger, less lipophilic compound which can be more easily excreted by the kidney. Since carbonyl containing compounds, such as naltrexone, are frequently hydrophobic and may be retained in tissues, their reduction to hydrophilic alcohols and subsequent conjugation are critical to their elimination (Felsted and Bachur, 1980a). The metabolism of xenobiotic ketones such as naloxone and naltrexone in mammals occurs primarily in the cytosol of liver and kidney, although metabolic activity can occur in many other tissues (Felsted and Bachur, 1980b). As the major pathway of metabolism of naltrexone in man is the reduction of the carbonyl group at C-6, this process will be described more fully below.

### **1.5.1.2. Carbonyl Reduction**

The reduction of an aldehyde or ketone moiety in a drug may not affect dramatically the pharmacological activity of the molecule. Reduction does however alter the physico-chemical characteristics of the molecule and as a result creates secondary effects on the lipid-aqueous phase distribution. This can alter the pharmacological distribution of the molecule produced by altering the intracellular distribution, tissue distribution, membrane permeability and membrane and protein binding of the molecule (Felsted and Bachur, 1980b). A more direct effect of the reductases on drug activity is the formation of a chiral carbon, and determination of a stereochemical configuration. The stereochemistry of the product, in the present context the metabolites 6 $\alpha$ - or 6 $\beta$ -naltrexol formation from naltrexone, which is determined directly by enzyme specificity, may drastically affect the product's action (Felsted and Bachur, 1980b).

A number of purified ketone reductases from the cytosol of tissues from various animals show at least 95% preference for NADPH over NADH as the cofactor, with optimal reductase activity between pH 5.0 and 5.4. Dihydromorphinone (e.g. naloxone and naltrexone) reductases have been identified in soluble liver extracts of pig, cow, guinea pig, rat, horse, rabbit, chicken and turkey (Felsted and Bachur, 1980b).

Kume and co-workers (1999) demonstrated a variant allele of the DD4 (AKR1C4) enzyme (DD4<sub>(S145C/L311V)</sub>), with approximately one third the normal catalytic activity towards naltrexone. (Kume et al., 1999). They suggested that this could account for the intersubject variabilities of the  $K_m$  and  $K_{cat}$  values found in an earlier study (Ohara et al., 1995). These differences in metabolism between individuals could result in large variability in plasma and brain naltrexone and 6β-naltrexol concentrations, and hence, could have an effect on drug efficacy, patient compliance and willingness to stay on naltrexone treatment.

The reduction of naltrexone and other drugs with a ketone group, was investigated in human liver cytosol by Ohara and colleagues in 1995. This group investigated the activities of carbonyl reductase, aldehyde reductase and three dihydrodiol dehydrogenases, and determined formation of the alcohol products, but did not quantify the end-products. The formation was calculated by measuring the rate of conversion of NADPH to NADP. The researchers found that all three dihydrodiol dehydrogenases reduced naltrexone, but not the aldehyde dehydrogenase or the ketone reductase, and that only 6β-naltrexol was formed by these enzymes. The specific activities for the enzymes were 100, 67 and 52 mU/mg for DD1 (AKR1C1), DD2 (AKR1C2) and DD4 (AKR1C4) respectively, with  $K_m$  values ranging from 0.19-1.6 mM (Ohara et al., 1995). The authors concluded that as dihydrodiol dehydrogenases are simply additional activities of 3α- and 17β-hydroxysteroid dehydrogenases, which are also associated with carbonyl reductase activity, these enzymes play an important role in the reduction of several ketone-containing drugs, including naltrexone and naloxone (Ohara et al., 1995).

More recently, Breyer-Pfaff and Nill (2004) incubated naltrexone with enzymes purified from human liver cytosol (gel filtration, ion exchange chromatography and chromatofocusing) in an NADPH-generating system at 37°C. The enzymes were the aldo-keto reductases AKR1C1, AKR1C2 and AKR1C4, and the carbonyl reductase CR I. The end-products were quantified with HPLC, and the kinetic parameters were calculated using the Michaelis-Menten equation by non-linear least-squares regression. They found that naltrexone was not reduced to 6β-naltrexol by CR, but that the AKR enzymes reduced naltrexone with  $K_m$  (mM) values of  $1.4 \pm 0.1$  (AKR1C1, n=3),  $0.13 \pm 0.06$  (AKR1C2, n=3) and 0.037 and 0.029 mM (AKR1C4, n=2), making the latter the predominant cytosolic enzyme involved (Breyer-Pfaff and Nill, 2004).

The effect of various inhibitors specific for particular enzymes has not been studied at this

stage, but Ahmed *et al* (1979), found that rabbit liver ketone reductase enzymes (which reduced naltrexone), were inhibited by the flavonoid quercetin (0.1 mM), but not by pyrazole (10 mM) or phenobarbital (1mM), both potent inhibitors of aldehyde reductases. Also no information is available on the effect of naltrexone on enzymes involved in the metabolism of endogenous ketone-containing compounds such as the sex-steroids testosterone and oestrogen and related compounds.

### **1.5.1.3. Other metabolic pathways**

In humans naltrexone and 6 $\beta$ -naltrexol undergo extensive glucuronide conjugation by glucuronosyl transferase enzymes in the liver, and O-methylation at the 3 position to form 3-methoxy-6 $\beta$ -naltrexol (Wall and Brine, 1981) by catechol-O-methyl transferases (COMT) found in brain, liver and red blood cells. Hydroxylation has also been shown to occur at the 2-position to give 2-hydroxy-3-methoxynaltrexol (HMN) (Verebey *et al.*, 1975), as shown in Figure 1.3.

It has also been reported that noroxymorphone is possibly formed in small quantities, by N-dealkylation of naltrexone (Ludden *et al.*, 1978). This is thought to be responsible for the agonistic actions of naltrexone observed by some researchers in human studies (Martin *et al.*, 1973; Resnick *et al.*, 1974; Verebey *et al.*, 1976). The enzymes responsible for this possible N-dealkylation have not been reported. There have been no studies examining the metabolism of naltrexone by the cytochrome P450 (CYP) family of enzymes.

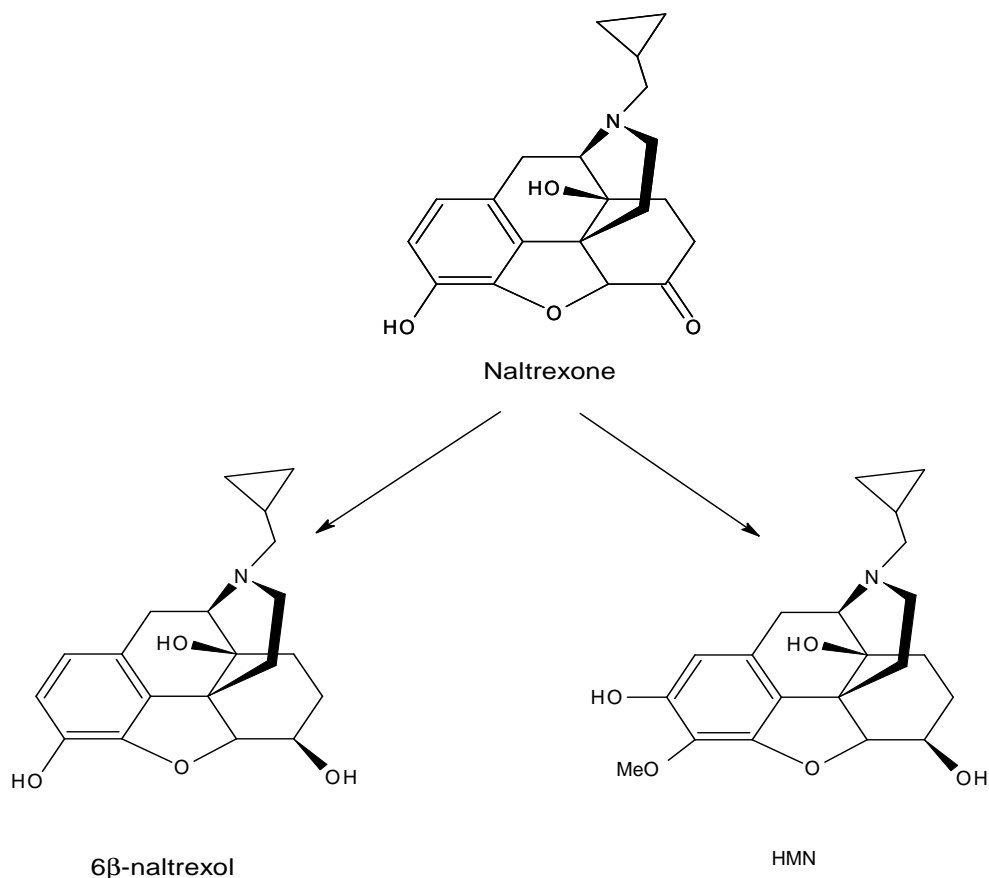


Figure 1.3. The metabolic pathways of naltrexone in humans. HMN is 2-hydroxy-3-O-methyl-6 $\beta$ -naltrexol, a minor metabolite in humans. Both naltrexone and 6 $\beta$ -naltrexol also undergo extensive glucuronide conjugation.

#### 1.5.1.4. Species variation of naltrexone metabolism

The metabolism of naltrexone shows large species variation. In humans, Chatterjie and colleagues (1974) extracted pooled urine samples from patients receiving 100-200 mg naltrexone per day orally. Whereas the major metabolite in man of naloxone is the glucuronide at the C-3, this group reported the reduced product with the 6 $\beta$ -hydroxy epimer as the major metabolite of naltrexone in humans, using MS, IR, NMR and TLC for identification (Chatterjie et al., 1974). These researchers also conducted *in vitro* experiments using chicken liver homogenates and found the chicken produced the 6 $\alpha$ -OH reduced product. The 6 $\beta$ -OH epimer was not detected (Chatterjie et al., 1974).

Further evidence of species variation of naltrexone metabolism was shown by Malspeis *et al* (1975). Urine and bile samples from monkeys, dogs, rabbits, guinea pigs, rats, mice and one human given single doses of naltrexone were examined qualitatively after hydrolysis for the presence of 6 $\beta$ - and 6 $\alpha$ -naltrexol. 6 $\beta$ -naltrexol was the major reduction product of man, Susan J Porter, PhD Thesis, 2010

monkey, guinea pig and rabbit, while trace amounts were detected in the other species. 6α-naltrexol was shown in only trace amounts from monkey and guinea pig urines, and not at all in the other species (Malspeis et al., 1975).

In a separate study, [15,16-3H] naltrexone was administered subcutaneously to rats, and naltrexone and its metabolites were separated by TLC, and the levels of radioactivity were measured. The authors found no 6α- or 6β-naltrexol in the plasma, and only very small amounts of 6β-naltrexol were found in the brain (Misra et al., 1976).

Further to this, using liver tissue from guinea pig, monkey and rat, Malspeis and co-workers (1976) showed that both 6α- and 6β-naltrexol were produced by the monkey and the guinea pig. In the monkey liver the reduction product was predominantly 6β-naltrexol, whereas in the guinea pig, the 6α-epimer predominated. A small amount of 6β- and no 6α-naltrexol was produced by the rat liver (Malspeis et al., 1976).

## **1.6. Pharmacokinetics of Naltrexone**

Despite the fact that naltrexone has been in clinical use as a pharmacotherapy in opioid dependence for almost 20 years, information regarding the disposition of naltrexone still appears to be conflicting and confusing. Naltrexone differs from naloxone in having a longer duration of action, a property attributed in part to 6β-naltrexol, (Cone et al., 1974) the major metabolite in humans (Gold, 1982; Inturrisi, 1976) as shown in Figure 1.2.

### **1.6.1.1. Naltrexone Absorption**

When taken orally, naltrexone is rapidly and almost completely absorbed, with very little of the unchanged drug (0.034%) reported being excreted in the urine (Cone et al., 1974; Meyer et al., 1984), and is 95% metabolised in the liver (Kleber, 1985). Cone and colleagues measured in urine at least 70% of the administered dose in healthy volunteers as metabolites, indicating that the absorption of naltrexone after oral administration is likely to be high, if not complete (Cone et al., 1974).

Verebey *et al* (1976) reported peak plasma naltrexone concentrations in humans approximately one hour after an oral dose of 100 mg, whereas the  $T_{max}$  (time taken to reach maximum plasma concentration) for 6β-naltrexol was two hours post-naltrexone. In that study the mean (n=4)  $C_{max}$  (maximum concentration) values after an acute 100 mg oral dose

of naltrexone were  $43.6 \pm 29.9$  ng/ml for naltrexone and  $87.2 \pm 25.0$  ng/ml for 6 $\beta$ -naltrexol. Both naltrexone and 6 $\beta$ -naltrexol were detectable in the plasma 24 hours post-naltrexone administration, but there was no accumulation of naltrexone or the metabolite 6 $\beta$ -naltrexol with chronic dosing of 100 mg/day of naltrexone (Verebey et al., 1976). Meyer *et al* (1984) using reversed-phase HPLC with electrochemical detection (ECD), reported mean  $T_{\max}$  values of 0.95 and 0.91 hours for naltrexone and 6 $\beta$ -naltrexol, respectively, with  $C_{\max}$  concentrations of 8.55 and 99.3 ng/ml for the two compounds following a single oral dose of naltrexone in 24 healthy, young volunteers.

After oral administration of naltrexone in humans, plasma concentrations of 6 $\beta$ -naltrexol have been reported to be approximately four times (Wall et al., 1981) and thirty times higher (Meyer et al., 1984) than naltrexone. It has been proposed (Inturrisi, 1976) that in humans the prolonged duration of action of naltrexone in blocking the effects of mu receptor agonists is contributed to, at least in part, by 6 $\beta$ -naltrexol.

Ferrari *et al* (1998), conducted serum concentration- time course studies of naltrexone and 6 $\beta$ -naltrexol in thirteen heroin dependent people on chronic naltrexone treatment. In six of these patients studied once during long-term treatment (11-29 months), after a 100 mg dose of naltrexone the values for the parent drug and the metabolite were obtained in serum. Values for  $T_{\max}$  were  $73 \pm 37$  minutes for both compounds, which agrees with the result obtained by Verebey *et al*, 1976;  $C_{\max}$  values of  $76 \pm 45$  and  $163 \pm 69$  ng/ml were obtained for naltrexone and 6 $\beta$ -naltrexol, respectively, and  $t_{1/2}$  (plasma elimination half-life) values were  $16 \pm 8$  and  $25 \pm 14$  hours for the parent compound and the metabolite, respectively. The other seven patients in the study were tested at the beginning of treatment and again after one month of naltrexone treatment. Although there were no reported differences between the two time points for each patient, there were marked differences between the patients. The researchers separated the seven patients into two groups showing distinct metabolic patterns. After one month, in one group (n=4), the  $C_{\max}$  (ng/ml) values were  $37 \pm 26$  and  $156 \pm 93$  for naltrexone and 6 $\beta$ -naltrexol, respectively, whilst the other group (n=3) had  $C_{\max}$  values of  $128 \pm 51$  and  $239 \pm 111$  for the parent drug and the metabolite. Various theories have been suggested for this apparent difference in metabolism by the authors, with differences in bioavailability thought to be the most likely explanation (Ferrari et al., 1998).

### 1.6.1.2. Bioavailability

One would anticipate that a drug with reported clearance values equal to or exceeding liver blood flow (see below) would have low oral bioavailability due to extensive hepatic first pass metabolism, and that other organs would be clearing the drug. Meyer *et al* (1984) calculated the bioavailability to be of the order of 5% (as one would predict), Kogan *et al* (1977) obtained values of 22%, and Wall *et al* (1981) obtained a figure of 40% (substantially higher than one would predict). The large difference could be due to the differences in assay sensitivities, different assay methods used (a mixture of tlc and liquid scintillation of radioactivity by Wall, HPLC by Meyer), or differences in subject populations (Meyer *et al.*, 1984; Kogan *et al.*, 1977; Wall *et al.*, 1981). These authors calculated these parameters based on the equations of Vaughan, which were not reported ((Vaughan, 1975) .

### 1.6.1.3. Excretion of naltrexone and metabolites

Following an oral dose of naltrexone of 50 mg, only 6.5% of the dose was excreted as unchanged drug in the faeces, further evidence for nearly complete absorption (Wall *et al.*, 1981). In an earlier study, faecal excretion of unchanged drug accounted for 2.1% and 3.5% after acute and chronic doses, respectively of 100 mg of naltrexone administered orally. The major excretory product was 6 $\beta$ -naltrexol, with small amounts of naltrexone and 2-hydroxy-3-methoxynaltrexol. There was no increase in excretory products with acid hydrolysis indicating excretion in the unconjugated form (Verebey *et al.*, 1976). These researchers also reported that less than 0.5% of the administered dose was excreted in the faeces as naltrexone, suggesting complete absorption of naltrexone.

### 1.6.1.4. Clearance

The renal clearance of naltrexone has been shown to be similar to that of inulin, suggesting the mechanism of renal clearance is by filtration only. The renal clearance ratio to inulin of 2 obtained for 6 $\beta$ -naltrexol indicates clearance is by tubular secretion (Wall *et al.*, 1981). These authors suggested that tubular secretion was also a major process in the elimination of conjugated naltrexone and 6 $\beta$ -naltrexol. In a study by Verebey *et al.*, (1976), the mean ( $\pm$ SD, n=4) renal clearance values of 67 ( $\pm$ 38) and 30 ( $\pm$ 14) ml/min for naltrexone following acute and chronic administration respectively, of 100 mg, indicate that naltrexone is partially reabsorbed. The corresponding values for 6 $\beta$ -naltrexol, were 318 ( $\pm$ 70) and 369 ( $\pm$ 116) ml/min for acute and chronically administered doses respectively, of 100 mg of naltrexone. The authors conclude that 6 $\beta$ -naltrexol is thus actively secreted by kidney tubules.

The total plasma clearance of naltrexone (non-conjugated plus conjugated) has been calculated as being 5.15 l/min after intravenous administration of 1 mg and 2.61 l/min following a subcutaneous dose of 5 mg; 5.70 l/min (oral clearance) after an oral dose of 50 mg (Wall, 1981; Wall et al., 1984). An estimated total plasma clearance value of 1.5 l/min was obtained by Meyer *et al* (1984) also after an oral dose of 50 mg (based on a theoretical estimate of bioavailability). The latter value was calculated using mean data of 24 subjects, but there is no reported deviation from the mean. All plasma clearance values were obtained using  $Cl_T = D/AUC_{\infty}$ , where D is the administered dose. As shown above, naltrexone has been shown to exhibit a late terminal phase. Where the plasma naltrexone concentrations are not measured for the late terminal phase, there will be an underestimation of AUC, resulting in an overestimation of total plasma clearance.

#### 1.6.1.5. Volume of Distribution

The apparent volumes of distribution of naltrexone (calculated using  $f \cdot D/AUC$ , where f is the systemic availability) have been reported as 16.1 l/kg after single doses, and 14.2 l/kg after repeated dosing, of 100 mg of naltrexone taken orally (Kogan et al., 1977).

#### 1.6.1.6. Half-life

A range of naltrexone plasma elimination half-life ( $t_{1/2}$ ) values have been reported in humans, from  $1.1 \pm 0.2$  hours after a single oral dose by Cone's group in 1974, to  $10.3 \pm 3.3$  (mean  $\pm$ SD) hours by Verebey *et al* in 1976 after oral doses of 100 mg, and 2.7 hours after an intravenous (IV) dose, and by Wall and colleagues in 1981 to be 2.73 hours after an iv dose, and 8.9 hours after an oral dose of 50 mg, although the inconsistencies between route of administration are not explained (Cone et al., 1974; Verebey et al., 1976; Wall et al., 1981). In the latter two studies, the inter-subject variability was reportedly high, (32% CV for Verebey, means only reported by Wall).

The half-life of 6 $\beta$ -naltrexol in humans has been reported to vary from 7.5 to 12.7 hours (Meyer et al., 1984; Verebey et al., 1976; Wall and Brine, 1981). The half-lives for 6 $\beta$ -naltrexol after acute and chronic dosing have also been reported as  $12.7 \pm 2.6$ , and  $11.4 \pm 2.0$  hours respectively (Misra, 1981). Meyer *et al* (1984) reported half-life values of  $3.57 \pm 2.62$  and  $12.24 \pm 2.43$  hours for naltrexone and 6 $\beta$ -naltrexol, respectively in 24 healthy males following an oral dose of 50 mg. Figure 1.4 shows the late terminal phase obtained by Meyer *et al* in this study.



NOTE:

This figure is included on page 30 of the print copy of the thesis held in the University of Adelaide Library.

*Figure 1.4. Plasma concentrations of naltrexone following oral naltrexone treatment, showing the late terminal phase (up to 24 hours). Taken from Meyer et al (1984).*

Ferrari *et al* (1998), reported  $t_{1/2}$  values of  $25 \pm 14$  and  $16 \pm 8$  hours for 6 $\beta$ -naltrexol and naltrexone, respectively, in six of thirteen heroin dependent people on chronic naltrexone treatment (50 mg /day) after a 100 mg oral dose of naltrexone. Blood samples were collected 20 and 40 min and 1, 2, 4, 8, and 24 hr after the 100 mg dose.

#### **1.6.1.7. Plasma Binding of Naltrexone**

Plasma binding of naltrexone has been reported as 20%, over a concentration range of 0.1-500 ng/ml, and was species independent (Ludden *et al.*, 1976). This figure has been widely used in pharmacokinetic calculations, but has not been verified.

#### **1.6.1.8. Naltrexone Slow Release Formulations (SRF)**

More widespread use of naltrexone as a pharmacotherapy for opioid and alcohol abuse and dependence has shown that while efficacious in both conditions, its usefulness as a treatment is limited by poor patient compliance. To circumvent this problem, a number of slow-release or depot formulations have been developed.

The single and multiple-dose pharmacokinetics of one such preparation (an injectable, microsphere-based sustained release delivery system) for deep intramuscular (im) injection were evaluated by Dunbar and colleagues (Dunbar, 2006). Subjects received either a single im injection of long-acting naltrexone (190 mg-n=12; 380 mg-n=12), or 4 x 380 mg every 28 days (n=12). Six subjects received placebo injections in a randomized, double-blind protocol. All groups showed plasma peaks after one day (due to drug diffusion from the surface of the microspheres), a secondary peak on day 2 (primarily due to polymer hydration), and sustained release over 30 days (due to polymer erosion). Both doses resulted in 6β-naltrexol concentrations greater than (approximately 2-fold) those of naltrexone, but the shapes of the two profiles were similar. The authors report that naltrexone pharmacokinetic parameters did not change with repeat dosing of 380 mg as the AUC (160 ng.d/ml) and absorption-drug release half-life ( $t_{1/2}$ ) (4.73 days) estimates were similar to those obtained after a single dose (144 ng.d/ml, 4.95 days) (Dunbar et al., 2006).

In an earlier study in alcohol-dependent subjects (n=16), naltrexone was administered as two 150 mg doses im on a single occasion (Galloway et al., 2005). Blood samples were collected at baseline and weekly for six weeks post-administration of naltrexone. Values for  $T_{max}$  were highly variable for naltrexone and 6β-naltrexol, ranging from 168-1008 hr and 468-1008 hr, respectively. The mean 6β-naltrexol to naltrexone ratios were 3.4 for  $AUC_{0-28}$  and 3.4 for  $AUC_{0-42}$ . The plasma 6β-naltrexol concentrations were approximately three times higher than those of naltrexone at all sampling time points. Both naltrexone and 6β-naltrexol were detectable up to the last day of sampling (day 42).

## **1.7. Pre-Clinical Pharmacology of Naltrexone and its Metabolites**

### **1.7.1.1. Potency and Receptor Binding Studies**

Naltrexone has been shown to act at mu, kappa and delta opioid receptors, with the highest affinity being for the mu receptor (Gianoulakis, 1993). The receptor binding constant ( $K_{RB} \times 10^{-9}$  M) using [ $^3$ H] dihydromorphine and 10,000g particulate fraction of rat brain homogenate was reported as 0.2 for naltrexone, compared to 1.0 for naloxone (Misra, 1981).

More recently, the binding characteristics and antagonistic effects of naltrexone, 6β-naltrexol and naloxone were evaluated in the cell membranes of human embryonic kidney cells (HEK) transfected with human  $\mu$ -opioid receptor (HEK-MOR), mouse  $\delta$ -opioid receptor (HEK-DOR) and human  $\kappa$ -opioid receptor (HEK-KOR). Receptor binding affinities were measured

by competitive inhibition of 0.5 nM [<sup>3</sup>H]diprenorphine (a nonselective opioid ligand). 6 $\beta$ -Naltrexol had the highest affinity for  $\mu$  ( $K_i$  1.4 $\pm$ 0.1 nM), then  $\kappa$  ( $K_i$  2 $\pm$ 1 nM), then  $\delta$  ( $K_i$  29 $\pm$ 14 nM) opioid receptors in this assay. The 6 $\beta$ -naltrexol binding affinities for  $\mu$  and  $\kappa$ -opioid receptors were 2- and 5-fold higher, respectively than naloxone ( $K_i$  7 $\pm$ 1 nM [ $\mu$ ], 4 $\pm$ 1 nM [ $\kappa$ ]), but 2-fold less potent than naltrexone ( $K_i$  0.5 $\pm$ 0.1 nM [ $\mu$ ], 1 $\pm$ 1 nM [ $\kappa$ ]), . 6 $\beta$ -Naltrexol was 3- to 4-fold less potent than naloxone ( $K_i$  8 $\pm$ 1 nM or naltrexone ( $K_i$  7 $\pm$ 1 nM) for the  $\delta$ -opioid receptor (Wang et al., 2007). In the same study, these antagonists were assessed for their effects on basal signalling of the receptors in the same membrane preparations (pre-treated with receptor-specific ligands) using <sup>35</sup>S-GTP $\gamma$ S binding as the measure of basal activity. As mentioned above, naltrexone and naloxone have been shown to be inverse agonists at  $\mu$ -opioid receptors, while 6 $\beta$ -naltrexol is a neutral antagonist. These results were confirmed in this study. Naloxone was shown to be an inverse agonist in the  $\delta$ -opioid receptor assay, naltrexone and 6 $\beta$  naltrexol were neutral. In the  $\kappa$ -opioid receptor assay system, naloxone again decreased basal activity, naltrexone did not, and remained neutral. However, 6 $\beta$ -naltrexol decreased basal activity when the membranes were pre-treated with U-69593 and U50,488, but not when pre-treated with morphine. Moreover, 6 $\beta$ -naltrexol inhibited the inverse agonist effect of naloxone in morphine-pre-treated  $\kappa$ -opioid receptor membranes. The authors postulated that the different pharmacological behaviour of antagonists may be a result of different affinities for various receptor conformations formed by constitutive activity of the receptor (Wang et al., 2007).

The different effects seen between naltrexone and naloxone, and 6 $\beta$ -naltrexol in the  $\mu$ -opioid receptor membrane preparations described above were not repeated in a more recent study using similar methodology in a heterologous expression system of C6 glioma cells together with HEK293 cells stably transfected with a FLAG-tagged mouse  $\mu$ -opioid receptor (Divin et al., 2009). In this study, in DAMGO-pre-treated G-protein activation did not decrease from basal values for any of these antagonists (i.e. they remained neutral). The  $K_i$  values for naltrexone and 6 $\beta$ -naltrexol were similar in both studies. Additionally, it has been shown in earlier studies that neutral antagonists inhibit the observable effects of inverse agonists (cf the inhibition of naloxone effects by 6 $\beta$ -naltrexol above). Naltrexone and 6 $\beta$ -naltrexol were co-administered in this study, with no difference in shift of the morphine dose-response curves compared to the agonists administered alone. The authors conclude that the  $\mu$ -opioid receptors in this preparation can not distinguish between naltrexone and 6 $\beta$ -naltrexol, and that both are acting as neutral antagonists.

The use of organ bath preparations of various animal tissues has long been an important pharmacological tool to assess potencies and antagonistic activity of agonists and antagonists respectively. One such tissue that has been frequently used in this type of study is the guinea pig ileum. Limited studies have been performed with naltrexone. Takemori and Portoghese (1984) tested the antagonistic activity of naltrexone principally against morphine, the prototypical  $\mu$ -opioid agonist in guinea pig ilea. This study used  $pA_2$  (Schild plots) to quantify the antagonistic activities of naltrexone and naloxone. Comparison of the  $pA_2$  values revealed that naltrexone was 3.5 to 5 times more potent than naloxone (Takemori and Portoghese, 1984). There are no reported studies comparing naltrexone and 6 $\beta$ -naltrexol in this preparation.

The functional antagonism of naltrexone was first determined in rats using counter-action of oxymorphone-induced (2 mg/kg sc) loss of righting reflex after subcutaneous injection of naltrexone by Blumberg and Dayton (1974). They showed the  $ED_{50}$  (the dose at which 50% of the animals responded to the antagonist) to be 0.023 mg/kg, making naltrexone twice as potent as naloxone ( $ED_{50}$  0.051 mg/kg). Oral antagonistic activity was measured the same way and naltrexone ( $ED_{50}$  0.98 mg/kg) was shown to be eight times as potent as naloxone ( $ED_{50}$  8.0 mg/kg), with the antagonism lasting three times as long (58 minutes and 169 minutes for naloxone and naltrexone, respectively (Blumberg and Dayton, 1974). These researchers also studied the antagonism of oxymorphone-induced Straub tail reaction in mice, and found naltrexone to be three times more potent than naloxone ( $ED_{50}$ 's 0.047 and 0.14 mg/kg, respectively) after subcutaneous injection. Additionally, the antagonism of oxymorphone-induced (2 mg/kg iv) respiratory depression was measured in rabbits. Following intravenous administration of the antagonist, the  $ED_{50}$  for naltrexone was 0.02 mg/kg compared to 0.045 mg/kg for naloxone (Blumberg and Dayton, 1974).

In contrast, 6 $\beta$ -naltrexol was only 1/85<sup>th</sup> as potent as naltrexone in achieving the same degree of antagonism of oxymorphone-induced Straub tail in mice (Chatterjee et al., 1975), and 1/26<sup>th</sup> as active as naltrexone in mice in preventing the Straub-tail reaction with morphine (30 mg/kg, subcutaneously), and approximately 9 times as long-acting (Misra, 1981). A study was also conducted measuring the number of jumps produced after administration of antagonist to morphine-dependent mice, in which the potency of 6 $\beta$ -naltrexol was 1/53<sup>rd</sup> that of naltrexone (Fujimoto et al., 1975).

As mentioned previously, it has been proposed that on long-term exposure to an agonist,  $\mu$ -opioid receptors exhibit a constitutively active form (activity in the absence of an agonist), and that naltrexone is an inverse agonist (can block this basal activity), whereas 6 $\beta$ -naltrexol is a neutral antagonist (no effect on basal activity). Recent studies have been conducted to ascertain if the antagonist potencies of naltrexone and metabolites are altered in agonist-dependent compared to agonist-naïve animals, using various paradigms of antagonism.

The antagonistic potencies of naltrexone, 6 $\beta$ -naltrexol and naloxone were compared in opioid-naïve and opioid-dependent mice with respect to antinociception (tail-flick) assay, locomotor activity and precipitation of withdrawal (Raehal et al., 2005). The antagonists were administered (icv or ip) 10 min prior to administration of 20 nM morphine (icv), and the mice were tested 20 minutes later in the tail-flick assay (55°C water). The ID<sub>50</sub> (the dose at which 50% of maximum inhibition was achieved) obtained in this assay for icv administration were 0.26 nM for naltrexone, 3.43 nM for naloxone and 2.32 nM for 6 $\beta$ -naltrexol. Administration of the antagonist ip resulted in ID<sub>50</sub> values of 0.22 mg/kg (naltrexone), 1.09 mg/kg (naloxone) and 1.0 mg/kg (6 $\beta$ -naltrexol). Thus the rank order of potency was naltrexone > 6 $\beta$ -naltrexol > naloxone (icv) and naltrexone > 6 $\beta$ -naltrexol=naloxone (ip). In the locomotor activity studies, the antagonists were administered ip, followed by sc morphine 30 mg/kg or vehicle. Naltrexone was 4 times more potent than naloxone and 10 times more potent than 6 $\beta$ -naltrexol in reducing the morphine-induced increase in locomotor activity, measured by the distance the mice travelled in the chambers.

In another part of the study, various dosing regimens of morphine were used to induce mild, moderate or severe opioid dependence. Withdrawal was precipitated with naltrexone (0.1 mg/kg ip) or 6 $\beta$ -naltrexol (1 mg/kg ip), and measured by counting the number of vertical jumps, body shakes, paw tremors and faecal pellets. Comparisons between naltrexone and 6 $\beta$ -naltrexol showed no differences in any of the parameters tested in animals with mild dependence. In animals with moderate and severe dependence, 6 $\beta$ -naltrexol precipitation of withdrawal resulted in significantly less faecal output and vertical jumps than naltrexone. Additionally, there were also less paw tremors in the animals with severe dependence when withdrawal was precipitated with 6 $\beta$ -naltrexol compared to naltrexone. These results indicate a decreased severity of withdrawal with precipitation by 6 $\beta$ -naltrexol. The authors concluded that if the mouse results are applicable to humans, 6 $\beta$ -naltrexol, as a neutral antagonist, may be a better alternative to naltrexone or naloxone in reversing life-threatening effects of opioid

overdose (by inducing less severe withdrawal profiles), and may be better tolerated in people recovering from severe dependence (Raehal et al., 2005).

The results reported above were supported in another mouse study, where the antagonist properties of naltrexone and 6 $\beta$ -naltrexone were assessed in morphine-dependent and morphine-naïve animals (Divin et al., 2008). The hypothesis in that study was that an inverse opioid receptor agonist that decreases basal signalling of a constitutively active receptor should precipitate a more severe withdrawal response than a neutral antagonist which can only precipitate withdrawal by displacement of an agonist, and that these differences are due to an apparent difference in their *in vivo* receptor antagonist potencies caused by differential access to agonist-occupied receptors. In this study, naltrexone and 6 $\beta$ -naltrexol antagonism of morphine-induced antinociception, time-course of antagonism of a long-acting agonist (BU72), and precipitation of withdrawal in morphine-dependent mice were assessed and compared. The authors report that at 10 mg/kg, both antagonists were equipotent in reversing morphine-induced antinociception in both the hotplate and tail flick assays in non-dependent animals. In BU72-pretreated mice, naltrexone and 6 $\beta$ -naltrexol (1 mg/kg sc) were equieffective in reversing antinociception in the tail-flick assay (50°C water), but the onset of antagonism by naltrexone was more rapid, and shorter-lasting ( $t_{1/2}$  11.3 $\pm$ 2.6 and 72.5 $\pm$ 15.7 min respectively, for naltrexone and 6 $\beta$ -naltrexol). In morphine-dependent animals (75 mg sc implant or 5x30 mg/kg doses 12 hours apart), there was a 10 to 100-fold difference in potency between naltrexone and 6 $\beta$ -naltrexol in eliciting withdrawal jumping behaviour. Only the highest dose of 6 $\beta$ -naltrexol (10 mg/kg sc) elicited the same number of jumps as 0.1 mg/kg naltrexone. The authors conclude that while these antagonists have equivalent *in vivo* receptor affinities, the differences observed in efficacy could be due to different abilities in reaching the site of action (central  $\mu$ -opioid receptors) and the rapidity of access needed to precipitate a robust withdrawal response (Divin et al., 2008).

In another study (Sirohi et al., 2009), the antagonist potencies of naltrexone, naloxone and 6 $\beta$ -naltrexol were compared in mice in tests of antinociception using the tail-flick (light beam) assay and prevention of lethality in non-dependent animals. Both assays used fentanyl as the agonist (100  $\mu$ g/kg for antinociception, 80 mg/kg for lethality). All drug administrations were subcutaneous. The relative potencies obtained for the antagonism of fentanyl-induced antinociception were naltrexone (17) > naloxone (4) > 6 $\beta$ -naltrexol (1). Similar relative potencies were obtained in the lethality study, with naltrexone (14) > naloxone (2) > 6 $\beta$ -

naltrexol (1).

To assess the severity of antagonist-induced withdrawal in the afore-mentioned study, mice were made dependent with either fentanyl (1 mg/kg/day via osmotic mini-pump) and after 72 hours administered saline or naltrexone (0.01-1.0 mg/kg), naloxone (0.01–10 mg/kg), or 6 $\beta$ -naltrexol (2.0-200 mg/kg); and morphine (25 mg pellet sc) or fentanyl (as above), and after 72 hours injected with saline or 6 $\beta$ -naltrexol (0.5-2.0 mg/kg), and then naloxone (1.0 mg/kg) 70 minutes later. The mice were observed for withdrawal jumping immediately after antagonist injection (naloxone in the latter paradigm) for 15 minutes. The graded relative potencies of the three antagonists in precipitating withdrawal jumping were naltrexone (1489) > naloxone (476) > 6 $\beta$ -naltrexol (1). 6 $\beta$ -Naltrexol dose-dependently decreased naloxone-induced withdrawal jumping in morphine-dependent mice, and abolished naloxone-induced withdrawal jumping in fentanyl-dependent mice. The authors reasoned that these results provide further evidence for the inverse agonism properties of naltrexone and naloxone, and the ability of 6 $\beta$ -naltrexol to block the effects of naloxone is further proof that it is a neutral antagonist, and as such may have clinical advantages over the inverse agonists in the treatment of opioid overdose and dependence (Sirohi et al., 2009).

A study was also conducted in monkeys to assess variations in antagonist potencies of naltrexone, 6 $\beta$ -naltrexol and 6 $\alpha$ -naltrexol in morphine-naïve and morphine-dependent (3.2 mg/kg daily for one year) animals. A discriminative stimulus paradigm was used. The rank order of potency for the antagonists was naltrexone > 6 $\alpha$ -naltrexol > 6 $\beta$ -naltrexol in both treatment groups, and the ED<sub>50</sub> were similar for all compounds, irrespective of treatment. The authors concluded that constitutive basal signalling induced by long-term morphine treatment, did not affect the antagonist potency of naltrexone (an inverse agonist) or 6 $\alpha$ - and 6 $\beta$ -naltrexol (neutral antagonists) (Li et al., 2008).

## **1.8. Clinical Pharmacology of Naltrexone**

### **1.8.1. Toxicity of Naltrexone**

Toxicity studies of naltrexone in humans at doses as high as 300 mg daily for 36 months, show that it is well tolerated. In one study naltrexone was administered in incremental doses from 100-800 mg/day over a number of weeks. Neither naltrexone nor its metabolites were detected two weeks after the 800 mg dose, indicating that naltrexone is completely eliminated from the body (Verebey et al., 1980).

Increased levels of the liver transaminases ALT and AST have been reported in some studies (Landsberg et al., 1976; Resnick et al., 1974), whereas one study found no increase in ALT or AST levels over this time period in nine of ten patients (Sax et al., 1994). The patient with the increased transaminase levels remained on a dose of naltrexone of 200 mg/day, and the liver enzyme levels returned to baseline (Sax et al., 1994). The side effects reported in one of the above studies were sluggishness, restlessness, nausea and abdominal pain (Resnick et al., 1974). Toxicity studies have not been conducted however, in the alcohol-dependent population, and in particular those people who have some degree of liver damage as evidenced by abnormal liver function, and blood cell morphology.

A study conducted in 1997 however, on moderate to heavy social drinkers investigated the association between naltrexone biotransformation and reported side-effects. Naltrexone and 6 $\beta$ -naltrexol concentrations were measured in urine three hours after a 50 mg dose of naltrexone. In 13 of the 24 subjects, no side-effects were reported. The side-effects reported by the remaining 11 subjects were headache (45%), nausea (18%), anxiety (36%), and spontaneous erection (45%). Interestingly, the group reporting side-effects had significantly higher (Mann-Whitney U test) 6 $\beta$ -naltrexol concentrations in urine than those who reported no side-effects. While the overall naltrexone and 6 $\beta$ -naltrexol concentrations are reported, the values for each of the groups in relation to side-effects are not. The plasma concentrations of naltrexone and 6 $\beta$ -naltrexol were not measured, so the significance of the side-effect profile reported is unknown (King et al., 1997).

Teratogenicity studies in animals, including rats, guinea pigs and dogs, showed effects of naltrexone on the equivalent of the normal human dose of 50 mg/day (Braude and Morrison, 1976). There are no data on the effects of long-term administration of naltrexone, or interactions (if any) between naltrexone and other drugs. Carcinogenicity studies have also not been reported, nor is it known what the long-term effects of naltrexone administration in children are, which is important as naltrexone has been trialled as a treatment for autism in children (Bouvard et al., 1995), (Gonzalez et al., 1994).

### **1.8.2. Naltrexone Pharmacotherapies**

Naltrexone was approved by the American Food and Drug Administration (FDA) in 1995 and in 1999 by the Therapeutic Goods Administration (TGA) of the Department of Health and Ageing in Australia, for use as a pharmacotherapy for ethanol-dependent individuals,



Studies in animals have shown that opioid agonists such as morphine have a biphasic effect on preference for alcohol, whereby low doses (0.5-10 mg/kg) increase, and high doses (>10 mg/kg) decrease alcohol consumption (Reid and Hubbell, 1992). The latter effect may be a non-specific consequence of general CNS depression caused by the high dose of opioid receptor agonists used. Various theories have been put forward to explain the role of opioids in ethanol dependence, which propose that ethanol increases the activity of the endogenous opioid system (Berg et al., 1992; Reid and Carpenter, 1992). This system is therefore believed to be important in mediating ethanol's reinforcing effects and excessive ethanol consumption, although the mechanism by which opioids (endogenous and exogenous) produce euphoria, tranquillity and other alterations in mood, is not entirely clear.

Further evidence for an opioidergic role in ethanol dependence is the finding that the opioid antagonists naloxone and naltrexone decrease alcohol consumption/preference in a dose-related manner in animals (Myers et al., 1986; Samson and Doyle, 1985). This effect has been shown to be centrally mediated (Hubbell et al., 1986; Linseman, 1989), but as yet the opioid receptor subtype(s) involved have not been determined.

Naltrexone has been used since 1984 as an adjunctive treatment in opioid addiction. In opioid dependent humans, oral naltrexone causes an acute abstinence syndrome and is at least an order of magnitude more potent than naloxone (Gonzalez and Brogden, 1988). The earliest study into the use of naltrexone as a treatment for heroin dependence was conducted by Martin and colleagues in the early 70's (Martin et al., 1973). The efficacy of naltrexone in antagonising the effects of single doses of morphine and preventing the development of physical dependence to chronically administered morphine was assessed in nine prisoner post-addict subjects. Naltrexone was administered twice-daily as an oral dose of 15 mg to six subjects. One subject received 25 mg twice daily and two others were administered a once daily dose of 50 mg. All doses were administered in a cherry-flavoured vehicle and subjects and observers were blinded to the treatment. The subjects were challenged with subcutaneous morphine doses of 15 and 30 mg and their subjective state and pupil size were assessed. Once maintenance naltrexone dose levels had been reached, the subjects were given morphine four times daily in increasing dose levels until a stabilisation dose of 240 mg/day was achieved. This was attained by the sixth day and maintained for a further eleven days after which time the morphine was abruptly withdrawn, but the naltrexone was continued for a further ten days. The intensity of withdrawal was assessed as were vital signs and signs of abstinence. The subjects and observers also completed chronic-dose questionnaires.

The authors found that 15 mg of naltrexone administered twice-daily attenuated the effect of morphine on pupillary constriction by a factor of 20. Only one subject identified the morphine as a narcotic and then on only two occasions, and all reported a mild aversion to morphine whilst on all naltrexone doses. On withdrawal from the morphine all patients reported abstinence symptoms and although all felt slightly to moderately bad, none were sick. The authors concluded that an oral daily dose of 50 mg of naltrexone produced a level of antagonism in preventing the development of physical dependence on morphine in man comparable to that produced by 4 mg orally a day of cyclazocine without the dysphoria of the latter. Additionally, the euphorogenic effects of morphine were antagonised effectively with this dose (Martin et al., 1973).

In addition to being an adjunct in the treatment of opioid and alcohol dependence, naltrexone has been trialled for a diversity of disease states and syndromes. These include treatment for pruritus (Peer, 1996), Bergasa *et al* (1995); autism (in children naltrexone is thought to decrease hyperactivity, obesity and irritability) (Gonzalez, 1994); treatment for repetitive self-injurious behaviour (Barrett, 1989); reduction in levodopa-induced dyskinesias (Manson, 2001), all with varying levels of benefit. More recently, trials have been conducted in patients with Crohn's disease, irritable bowel syndrome and fibromyalgia (reviewed by (Leavitt, 2009), with positive results. Naltrexone has also been studied in other addictive disorders such as gambling (Crockford and el-Guebaly, 1998), (Kim and Grant, 2001).

In Australia, naltrexone hydrochloride (marketed as ReVia, 50 mg oral preparation) was approved for use in January 1999 specifically for use within comprehensive treatment programmes for alcohol dependence. It is also indicated as adjunctive therapy in the maintenance of formerly opioid-dependent patients who have ceased the use of opioids. At the time of writing (May 2010), these are still the only indications for the use of naltrexone in the clinical setting (MIMS Prescribing Information, 2009).

### **1.8.3. Opioid Detoxification**

Where opioid substitution is available, a prescribed opioid is substituted for the illicit substance and gradually the dose is decreased. Clonidine is used to alleviate withdrawal symptoms. Benzodiazepines are often used to relieve anxiety and insomnia. Detoxification can be achieved in a matter of days.

Naltrexone has been traditionally used as a maintenance pharmacotherapy. Following detoxification from opioids, as long as the opioid receptors are blocked by naltrexone, an opioid challenge will result in no euphoria, resulting in minimal reinforcement, allowing the dependent person to change their behaviour patterns and lifestyle, thus reducing the likelihood of relapse. The use of naltrexone for ultra-rapid opioid detoxification has generated significant publicity and controversy. The procedure involves administering naloxone and naltrexone under anaesthesia to allow for acute withdrawal to be completed within several hours, as opposed to days, followed by induction onto oral naltrexone for maintenance therapy. This treatment has been hailed as a “miracle cure” by the popular press (Simon, 1997). The controversy arose over improper management of heavily sedated or anaesthetised patients by some researchers (Simon, 1997), and lack of assessment of efficacy and cost-effectiveness of this treatment compared to conventional detoxification methods. Several studies have been conducted since, and while rapid detoxification resulted in increased numbers of opioid-dependent patients being inducted onto naltrexone, the relapse rates and low compliance on maintenance therapy were no better in the long-term than standard detoxification (McGregor et al., 2002; Bell et al., 1999).

#### **1.8.4. Low Dose and Ultralow Dose Naltrexone Therapy**

##### **1.8.4.1. Opioid Dependence**

As shown above, the usual dose for naltrexone in the treatment of opioid or alcohol dependence is 50 mg daily or 100 mg three times weekly, orally. In some of the disease states mentioned above, the doses of naltrexone have been low (1-5 mg) or ultralow (<1 mg) per day (Leavitt, 2009). In a study on dorsal root ganglion (DRG) neurons in culture, Crain and Shen showed that when ultralow concentrations of naltrexone (picomolar) were co-administered with morphine, the tolerance to the inhibitory action potential duration effects of morphine (high dose), and the supersensitivity of excitatory action potential duration-prolonging effects of morphine (ultralow dose) were prevented. Furthermore, when low doses of naltrexone (10  $\mu$ g/kg) were co-administered with morphine (10-100 mg/kg), to mice, the antinociceptive effects of morphine were enhanced, but the development of withdrawal symptoms was attenuated in chronic and acute physical dependence assays). The authors conclude that low and ultralow doses of naltrexone may be useful in the treatment of opioid dependence in humans (Crain and Shen, 1995).

A randomised, controlled trial of ultralow doses (0.05 and 0.5 mg/day), compared to the usual dose (50 mg/day) naltrexone in opioid-dependent humans was conducted to test differences

between the doses primarily in retention in treatment and heroin use, and secondly side effect profiles and craving over a six month period. To obtain a 25% improvement in retention over six months, a total sample of 180 people was required (60/group). At the six month follow-up, in the initial 66 people enrolled in the study (approximately 20/group), there was no relationship between naltrexone dose and retention in treatment, abstinence from heroin use, cravings or reported side effects. On the basis of these results the trial was discontinued, as the retention rates were low, and a number of the people elected to change their dose to the 50 mg/day standard treatment (Rea et al., 2004).

#### **1.8.4.2. Pain Management**

The major use for opioids in humans is in the management of pain. The ideal analgesic would alleviate pain without any adverse effects. In light of the early studies by Crain and Shen described above, many studies have been conducted to assess the effect of low and ultralow doses of antagonists administered in combination with agonists on nociception and pain, and to counteract the hyperalgesia associated with the chronic use of opioid agonists (Abul-Husn et al., 2007).

In a rat study, the selective  $\mu$ -opioid receptor antagonists CTAP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub>) (0.001 ng) and CTOP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>) (0.01 ng), increased the acute morphine (15  $\mu$ g) effects in both the tail-flick and paw pressure tests. Additionally, these antagonists inhibited or reversed the loss of potency seen with chronic morphine administration (Abul-Husn et al., 2007).

In an earlier rat study, administration of intrathecal (0.05 and 0.1 ng), or systemic (10 ng/kg ip) doses of naltrexone was shown to augment morphine-induced antinociception in the tail-flick assay. Additionally, chronic administration of low doses (0.005 and 0.05 ng it, or 10 ng/kg ip) naltrexone with morphine (15  $\mu$ g it or 15 mg/kg ip) over 7 days, inhibited the development of tolerance to morphine and partially restored morphine potency in animals previously showing tolerance (Powell et al., 2002).

Conditions where opioids alone have been of limited effectiveness are fibromyalgia and neuropathic pain (Leavitt, 2009). Studies of neuropathic pain in rats, caused by spinal nerve ligation or chronic constriction injury (CCI), have shown that activated spinal cord glia (possibly initiated by toll-like receptor 4 [TLR4] activation (Hutchinson et al., 2008), and elevated spinal dynorphin levels (possibly acting via NMDA receptor complex) (Malan et al.,  
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2000) are implicated in tactile allodynia and thermal hyperalgesia.

An interesting finding in a rat study of neuropathic pain was that the non-neuronally-active stereoisomer (+) of naloxone and naltrexone (60  $\mu$ g it), and systemically administered (+)- and (-)-naloxone could reverse CCI-induced mechanical allodynia (Hutchinson et al., 2008).

Clinical studies looking at combination therapies (agonist plus low/ultralow dose antagonist) in human trials of neuropathic pain were based on these and other studies.

### 1.9. Summary

As can be seen above, a large number of preclinical and clinical studies have been conducted to characterise the pharmacology of naltrexone. At the time the studies reported in this thesis were conducted, little work had been done on the *in vitro* and *in vivo* potencies of 6 $\beta$ -naltrexol, although it was known to be the major metabolite of naltrexone in humans, and thought to contribute to the longer duration of action of naltrexone compared to naloxone. The longer duration of action of this metabolite, even if not as potent as the parent compound, should be taken into consideration in designing dosing regimens. Additionally, there was a paucity of data on the kinetics of the formation of 6 $\beta$ -naltrexol from naltrexone *in vitro*, and the possible inhibitors of this formation.

There are major discrepancies between studies in the pharmacokinetics of naltrexone. In particular, it is important to determine a reliable estimate of the half-life after both intravenous and oral dosing (this could influence dosing interval), as well as the total systemic clearance and bioavailability, since these could determine maintenance dose rates. If differences in bioavailability between patients is the source of much of the variability found in AUC and C<sub>max</sub> values, it is of importance to ascertain the reasons for the differences. Most of the reported studies were conducted nearly 30 years prior to this one, and analytical techniques have improved markedly since that time.

In order to assess the pharmacokinetics of naltrexone in patient populations, a simple reliable method for the quantification of both naltrexone and 6 $\beta$ -naltrexol was required in a variety of biological fluids.

### 1.10. Aims of the Current Project

The aims of this study were to:

- 1) Pharmacologically characterise naltrexone and the human metabolite 6 $\beta$ -naltrexol by:
  - a) synthesising the metabolite 6 $\beta$ -naltrexol from the parent compound naltrexone.
  - b) assessing the potencies of naltrexone and 6 $\beta$ -naltrexol compared to naloxone *in vitro*, in the guinea pig ileum,
  - c) determining the antagonism of morphine antinociception by naltrexone and 6 $\beta$ -naltrexol compared to naloxone *in vivo* in the mouse .
  
- 2) Develop and validate assays to quantify naltrexone and 6 $\beta$ -naltrexol in human plasma, urine, saliva and cytosolic preparations using High Performance Liquid Chromatography, to be used in the:
  - a) determination of the kinetics of the enzyme(s) involved in the metabolism of naltrexone in human liver cytosol preparations
  - b) identification of inhibitors of the formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosol preparations.
  - c) measurement of naltrexone and 6 $\beta$ -naltrexol in biological fluids
  
- 3) Further characterise the pharmacokinetics of naltrexone and 6 $\beta$ -naltrexol in healthy individuals following a single oral and a single intravenous dose of naltrexone.

## 2. Synthesis of 6 $\beta$ -naltrexol from naltrexone

### 2.1. Introduction

As stated in 1.3.6.3. above, 6 $\beta$ -naltrexol is the 6-keto reduction product and major human metabolite of naltrexone. When this study commenced (1997), 6 $\beta$ -naltrexol was not commercially available. In order to obtain pure compound, most of the early published studies used 6 $\beta$ -naltrexol obtained from either enzymatic reduction from naltrexone in animal liver extracts (Fujimoto *et al.*, 1975), or extractions of urine from guinea pigs (Cone *et al.*, 1974) or human volunteers dosed with naltrexone. For example, Chatterjie *et al* (1974) extracted urine (five gallons in total) from volunteers administered naltrexone 100-200 mg/day (time-frame not stated) and Verebey *et al* (1975) extracted 500 ml of urine from a volunteer taking 160 mg/day orally. In the former study, the urine samples were made alkaline with NaOH and NaHCO<sub>3</sub> and extracted with ethyl acetate. Further extraction and purification of the product yielded a compound with an R<sub>f</sub> (retention factor, distance travelled by compound/solvent front) value of 0.49 when spotted on silica gel plates with an ethyl acetate/hexane/ethanol/ammonia (60:25:14:1) solvent system; a molecular weight of 343 as determined by mass spectroscopy; and lack of an absorbance peak in the carbonyl region on infra-red spectral analysis. This product was deemed to be the 6-keto-reduction product with the hydroxyl group in the beta orientation as determined by proton NMR (Chatterjie *et al.*, 1974).

Chatterjie *et al* (1975), also attempted to reduce naltrexone using lithium tri-*sec*-butylborohydride as the reducing agent, but this resulted in the formation of 6 $\alpha$ -naltrexol only. Other methods of reduction of naltrexone have been used and although the yields were high (98%), they usually gave mixtures of products. For example, one such method used sodium borohydride as the reducing agent, and a chloroform extraction procedure, but this resulted in a mixture of 6 $\alpha$ :6 $\beta$ -naltrexol of 85:15 (Malspeis *et al.*, 1975). Ahmed and colleagues (1979) also used sodium borohydride (2 molar equivalents) to reduce naltrexone, but they did not report either yield or purity.

The reduction of naltrexone to 6 $\beta$ -naltrexol has been successfully accomplished by a stereospecific reduction of the carbonyl group at carbon 6, using formamidinesulfinic acid (thiourea dioxide) under a stream of nitrogen in an aqueous, alkaline medium at 85°C (Chatterjie *et al*, 1975). In that study, 6 $\beta$ -naltrexol was obtained in yields of greater than 88%, with no indication of the 6 $\alpha$ -naltrexol epimer. The reduction product (as the

hydrochloride salt) was recrystallised with 95% EtOH-CH<sub>3</sub>COCH<sub>3</sub> and was identified by infrared spectroscopy (lack of absorbance at 1700 cm<sup>-1</sup>, a broad peak at 3500-3100 cm<sup>-1</sup>), melting point (free base 188-190°C, hydrochloride salt 205-210°C), mass spectrum (70eV) m/e 343 (100%), and optical rotation of  $[\alpha]^{25}_D -133.8^\circ$  (c 1, H<sub>2</sub>O). The proton nuclear magnetic resonance spectrum of the compound exhibited a doublet centred at  $\delta$  4.54 (J=6 Hz) due to the 5 $\beta$  proton and a multiplet due to the 6 $\alpha$  proton in the region of  $\delta$  3.68-3.45 (Chatterjie *et al.*, 1975).

In a later study (Simon *et al.*, 1994), 6 $\beta$ -naltrexol was synthesised stereospecifically from dihydroisocodeine and dihydroisomorphine using the Mitsunobo reaction with greater than 90% yields. The purity of the compound was analysed using melting point, <sup>1</sup>H-NMR and MS data. The compound had a melting point of 205-208°C as the hydrochloride salt. The mass spectrometry data showed a molecular ion at 343, with a daughter ion at 302. The proton NMR had multiplets at  $\delta$  0.1- 0.9 (the cyclopropyl hydrogens) and  $\delta$  3.6 (the 6 $\alpha$ -hydrogen) and a doublet at  $\delta$  4.6 (the 5 $\beta$ -hydrogen).

The partitioning of compounds between oil and water (partition coefficient) gives an indication of the lipid solubility of the compound and therefore the ability to cross biological membranes such as the blood-brain barrier, *in vivo*. The octanol:water ratio for naltrexone has been previously examined, but has not been reported for 6 $\beta$ -naltrexol. Using the hydrochloride salt of naltrexone, the octanol:water partition coefficient was 45.60 at 20°C and 83.3 at 37°C. These values are significantly higher than those obtained for morphine sulphate of 6.03 and 6.23 at 20°C and 37°C, respectively (Kaufman *et al.*, 1975a). As previously stated, it has been shown that for basic drugs, it is more appropriate to use a buffered solution, such as phosphate buffer at pH 7.4 (the physiological pH) to measure the partition coefficients (Wang, 1980).

## 2.2. Aim

The aim of the present study was to produce sufficient 6 $\beta$ -naltrexol for pharmacological and analytical purposes, and this was achieved by reducing naltrexone to 6 $\beta$ -naltrexol using a modification of the method of Chatterjie *et al.* (1975). The purity and identification of the product was assessed by melting point, infrared spectroscopy, <sup>1</sup>H nuclear magnetic resonance, thin layer chromatography and mass spectroscopy. Additionally, the octanol:pH 7.4 buffer partition coefficient for naltrexone and 6 $\beta$ -naltrexol was determined, and compared to



naloxone, using a method previously employed in this laboratory for morphine and metabolites (Van Crugten, 1991).

## **2.3. Materials and Methods**

### **2.3.1. Chemicals**

The reduction of naltrexone was performed by myself in the Department of Chemistry, University of Adelaide under the guidance of Dr Marc Kimber and Dr David Ward. Naltrexone hydrochloride and formamidinesulfinic acid (thiourea dioxide) were obtained from Sigma Pharmaceuticals (St. Louis, Mo, USA). Other chemicals used were sodium hydrogen carbonate (NaHCO<sub>3</sub>), disodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) (BDH Chemicals, Kilsyth, Australia); HCl (Ajax); triethylamine (TEA) Prolabo, Paris, France. Ethyl acetate (HPLC grade), ethanol, hexane, hydrochloric acid (analytical grade) and methanol (HPLC grade) were purchased from BDH Chemicals, (Poole, UK). Chemicals used in the octanol:pH 7.4 buffer study were octanol (May and Baker, Dagenham, UK), Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub> (AnalaR, Merck, Kilsythe, Australia), naloxone hydrochloride and morphine sulphate (Sigma Pharmaceuticals). All other chemicals were of analytical grade quality and purchased from commercial sources.

### **2.3.2. Analytical equipment**

The infrared spectroscopy was performed on a Mattson FTIR analyser (ThermoOptik, Franklin, MA, USA) using nujol mulls of the compounds. Melting points were determined on a Kofler Hot Stage fitted with a Reichert microscope. Mass spectra (MS) were obtained on an electron impact VG ZAB-2HF spectrometer (Thermo Electron Corporation, Greenbush, USA) and recorded at 70eV. Proton nuclear magnetic resonance (NMR) spectra were determined in deuteriochloroform solutions containing tetramethyl silane (TMS). Spectra were recorded on an ACP-300 MHZ (Varian, Melbourne, Australia) spectrometer. Thin layer chromatography (TLC) was conducted using MERCK Silica gel 60 F-254 aluminium plates and visualised under UV light. The solvent system for the thin layer chromatography was ethyl acetate/hexane/ethanol/ammonia (60:25:14:1).

High pressure liquid chromatography (HPLC) comprised a Radial-Pak Cartridge phenyl column packed with 4  $\mu$ m phenyl 5 NVP4 $\mu$  (Waters, Milford, USA). It was inserted in a radial compression module (RCM 8x10, Waters), and the pressure was in the required mid-green pressure zone (approximately 2500 psi). The pre-column was packed with Bondapak C18/corasil (Waters) and linked with two precolumn filters (Scientific Systems Inc 250148, Susan J Porter, PhD Thesis, 2010

State College, PA, USA). The integrator was a Shimadzu C-R6A (Shimadzu Corporation, Kyoto, Japan), and detection was with an ultra violet detector either UVIDEC-100, (Shimadzu), or Jasco 875 UV, (Jasco, Tokyo, Japan). The UV detector wavelength was set at 220 nm and the range setting was 0.0025 AUFS. The mobile phase comprised 16% methanol, 0.2% triethylamine (TEA) and 50 mM KH<sub>2</sub>PO<sub>4</sub> at pH 3.0, and the flow rate was 1.5 ml/min. For each compound (naltrexone and synthesised 6 $\beta$ -naltrexol), 50  $\mu$ l of 20  $\mu$ g/ml in water was injected onto the column. In order to reduce the retention times, the mobile phase was altered to 16% methanol, 0.2% TEA, 50 mM KH<sub>2</sub>PO<sub>4</sub> and pH 3.0.

The same HPLC system was used for the octanol:pH 7.4 buffer analyses, with modifications to the mobile phase being 25 mM KH<sub>2</sub>PO<sub>4</sub>, at pH 5.4, with a flow rate of 0.8 ml/min. The mobile phase was filtered through 0.2  $\mu$ m filters under vacuum and degassed by sonication prior to use.

### 2.3.3. Naltrexone hydrochloride reduction

The synthesis was performed initially on a small scale, necessitating minor modifications to the method of Chatterjie *et al*(1975). Previously, in 1996, Dr Marc Kimber from the Department of Chemistry, University of Adelaide, had used this method to synthesise a small amount ( $\cong$  50 mg) of the free base. This method was to be used in the current experiment, but an attempt was made to convert the product to the hydrochloride salt and then recrystallise it, however, this was not successful as described below.

To 3.32 ml of water containing 42.5 mg NaOH was added 50 mg naltrexone HCl, then 57.3 mg formamidinesulfinic acid. This solution was stirred at 80-85 $^{\circ}$ C under nitrogen for one hour, then the mixture was cooled and the pH was adjusted to 9.8 with 4N HCl. As no precipitate was observed, the mixture was extracted twice with 20 ml of ethyl acetate, dried down and a white powder was obtained. To convert the product to the hydrochloride salt, it was dissolved in ethanol/acetone 1:1 (5 ml) and treated with 4 N HCl, which turned the solution yellow. The solvent/acid solution was dried under vacuum whence it became green and oily. Attempts to recrystallise the product were unsuccessful on this small scale, so it was decided to follow the method of Chatterjie *et al* (1975) more closely, however, the product would be recrystallised as the free-base.

Subsequently, 754 mg of naltrexone hydrochloride in 50 ml water (2 mM) was made alkaline with approximately 2 ml NaOH solution (640 mg/50 ml), which was further treated with 864

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mg of formamidinesulfinic acid (8 mM dissolved in the remaining NaOH solution). This solution was stirred for one hour at 82°C under a stream of nitrogen. After cooling, a white precipitate was formed, and the pH was adjusted to 9.8 with 4N HCl. The precipitate was filtered and washed with cold water to give a yield of 507.4 mg (67.3%), hereafter referred to as Product 1. As the yield was significantly lower than that obtained by Chatterjee *et al* (1975), the mother liquor was extracted with ethyl acetate (80 ml), which yielded a further 47.5 mg (5.9%) of a slightly more yellow powder, hereafter referred to as Product 2. This yield was still lower than the 88.5% reported by Chatterjee *et al* (1975). If the extracted product proved to be also 6 $\beta$ -naltrexol, the total yield would be 73.2%. Melting points, TLC, IR and <sup>1</sup>H NMR, and HPLC were performed on both products (products 1 and 2).

The above experiment was repeated, with the exception that the reaction was not performed under bubbling N<sub>2</sub>, but was flooded with N<sub>2</sub> at the commencement only, to give products 3 (332.1 mg, 44% yield) and 4 (146 mg, 19% yield). Results of analyses of these crude products (Products 3 and 4) showed that both were a mixture of products (Table 2.2), and an unsuccessful attempt was made to separate the products by flash chromatography; therefore these samples were not recrystallised and were discarded. A further reduction was done by the same method, with a new bottle of naltrexone hydrochloride, and products 5 and 6 were obtained (Table 2.1). Appearance and results of analyses of the products obtained are shown below. With the exception of Products 3 and 4, all products were recrystallised with 95% ethanol/acetone, and identified as previously stated.

#### 2.3.4. Octanol:pH 7.4 Buffer Study

The method employed in the current study was based on that used by Van Crugten *et al* (1991) used previously in this laboratory to examine the octanol:pH 7.4 phosphate buffer partition coefficients of morphine and metabolites. The octanol and the phosphate buffer (a combination of 67 mM Na<sub>2</sub>HPO<sub>4</sub> and 67 mM NaH<sub>2</sub>PO<sub>4</sub> to give pH 7.4) were initially saturated with each other. Four samples containing 5 ml of octanol and 5 ml of buffer were mixed for 4 hours at room temperature (22°C). After centrifugation at 3000 rpm for 20 min, the buffer and octanol phases were combined (approximately 1 ml of the interface solution was discarded).

Morphine sulphate, naltrexone hydrochloride, naloxone hydrochloride and synthesised 6 $\beta$ -naltrexol stocks (100  $\mu$ g/ml) were diluted in octanol-saturated phosphate buffer (pH 7.4) to give solutions of 10, 50 and 100  $\mu$ g/ml of the free base. Samples of 20  $\mu$ l of all of the above

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samples were injected onto the column as pre-equilibration controls. The partitioning of substrates into octanol was determined by rotary mixing 0.8 ml of the sample with 0.8 ml of the phosphate-saturated octanol for 24 hours at room temperature. The samples were centrifuged for 20 min at 3000 rpm and the upper octanol layer removed. A 20  $\mu$ l sample of the phosphate layer was injected onto the HPLC to obtain the post-equilibration data, which were compared to the pre-equilibration controls by comparison of the peak height ratios.

## **2.4. Results**

### **2.4.1. Naltrexone HCl reduction**

The mass spectrometry, infra-red spectroscopy, and proton NMR of Product 1 are shown in Figures 2.1, 2.2 and 2.4, respectively. For comparison, the proton NMR and infrared profiles of naltrexone are shown in Figures 2.3 and 2.5, respectively. The HPLC of Product 1 alone and Product 1 plus naltrexone are shown in Figure 2.6 (a) and (b), respectively, with the mobile phase modifications previously mentioned to reduce retention times. Tables 2.1 and 2.2 show the yields, appearance and chemical properties of the obtained products. For comparison the parent compound naltrexone hydrochloride and the 6 $\beta$ -naltrexol synthesised in 1996 are included in the tables. Products 5 and 6 (with a total yield of 78% 6 $\beta$ -naltrexol) show the molecular ion (m/e) to be 344 rather than 343 of Products 1 and 2 but the melting point data were similar. The peaks reported for the proton NMR are those corresponding to the protons attached to Carbons 5 and 6 only, and products 1, 2, 5 and 6 all showed the multiplet between  $\delta$ 3.51-3.61 of the  $\alpha$ -hydrogen of Carbon 6.

The HPLC data showed two peaks, the first at 13.5 min which accounts for approximately 3% of the total peak area of the chromatogram. This peak corresponds to 6 $\alpha$ -naltrexol when compared to a sample in the laboratory, (synthesised in 1996 by Dr Marc Kimber, see section 2.2.3), containing 85% 6 $\alpha$ -naltrexol and 15% 6 $\beta$ -naltrexol. Therefore, the reduction products 1-6 all contained between 1.7 and 3.6 % 6 $\alpha$ -naltrexol as calculated on the peak areas obtained by the integrator.. Products 3 and 4 also showed a peak at 18.5 min corresponding to the naltrexone starting product which accounted for 21% of the total area of the chromatogram for Product 3, and other peaks which corresponded to thiourea dioxide used in the reduction of naltrexone. Products 3 and 4 also showed a peak on the infrared spectrograms at 1718 corresponding to the C=O of the starting product naltrexone, while the proton NMR for these compounds showed numerous extra peaks. Due to these impurities the mass spectrometries were not performed for these products.

Table 2.1 Appearance, yield and melting points of products of naltrexone hydrochloride reduction. Note- MP = melting point

Compound	Appearance	MP (°C)	Yield (%)
Naltrexone HCl	White powder	168-170	
Naltrexone*	White powder	188-190	88.5
6 $\beta$ -naltrexol 1996	Brownish powder	188-190	Unknown
Product 1 1998	White fluffy powder	188-190	67.3
Product 2	Brown powder	188-190	5.9
Product 3	Brown powder	160-161	44.0
Product 4	White crystals	105-107	19.4
Product 5	White powder	188-194	72.4
Product 6	Brown crystals	188-190	5.6

\* Chatterjie et al, 1975

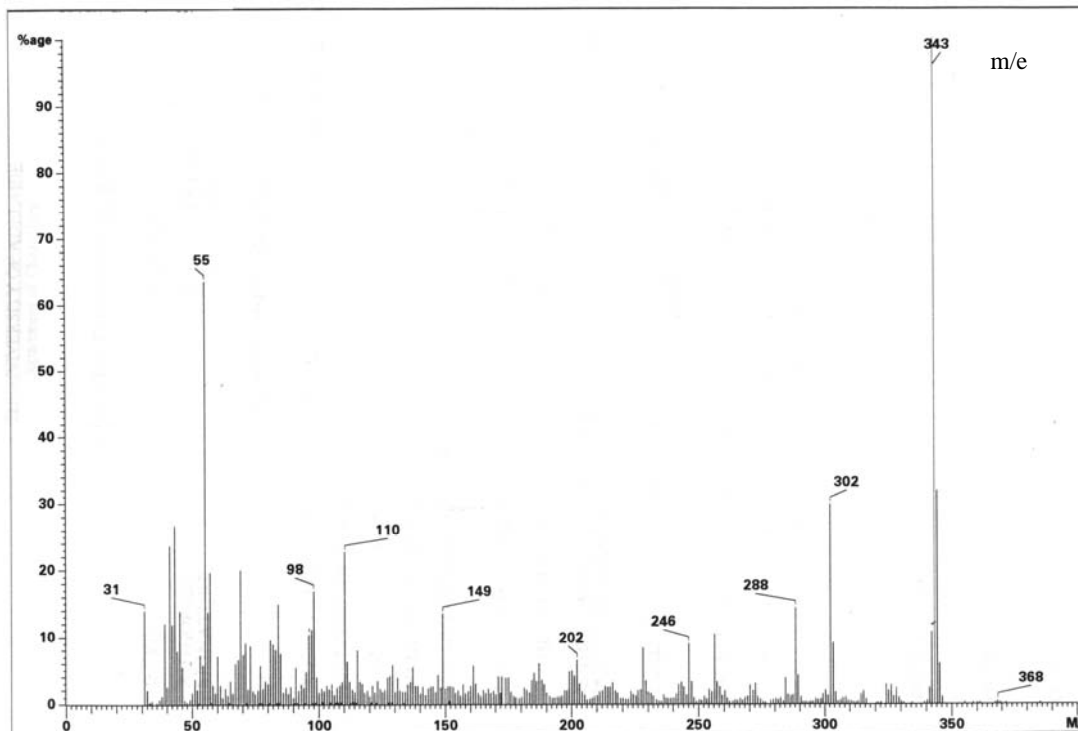


Figure 2.1 Mass Spectrometry of Product 1 Note: molecular ion 343

Table 2.2 Analyses of synthesised products following the chemical reduction of naltrexone hydrochloride.

Compound	tlc R <sub>f</sub>	IR cm <sup>-1</sup>	HPLC R <sub>t</sub>	MS ions	<sup>1</sup> H NMR
Naltrexone HCl	0.54	C=O 1715 O-H 3100-3400	18.55	**	$\delta$ 4.37(s) 5 $\beta$ H
Naltrexone <sup>&amp;</sup>	0.55	O-H 3100-3500	-	m/e 343	$\delta$ 4.54 5 $\beta$ H $\delta$ 3.68-3.45 6 $\alpha$ -H
6 $\beta$ -naltrexol 1996	0.24	No C=O O-H 3150-3351	13.5 23.5	m/e 343, 302, 256, 149, 129,	$\delta$ 4.54 -5 $\beta$ H $\delta$ 3.58-3.50 - 6 $\alpha$ H
Product 1	0.25 0.51*	No C=O O-H 3152-3383	13.5 23.4	m/e 343, 302, 288, 256, 149,	$\delta$ 4.52 5 $\beta$ H $\delta$ 3.54-3.61- 6 $\alpha$ H
Product 2	0.25 0.4*	No C=O O-H	13.55 23.48	m/e 343, 302, 256, 149, 129,	$\delta$ 4.50 5 $\beta$ H $\delta$ 3.55-3.59- 6 $\alpha$ H
Product 3	0.23, 0.54 0.79	C=O 1718	13.47 18.41 23.35	**	Very messy, mixture of products
Product 4	0.20	C=O 1719 Strong 1859	13.57 18.58 23.51	**	Very messy, mixture of products
Product 5	0.27 0.35*	No C=O O-H 3151-3384	# 9.813 15.458	m/e 344, 303, 256, 228	$\delta$ 4.51 5 $\beta$ H $\delta$ 3.52-3.57- 6 $\alpha$ H
Product 6	0.21 0.35*	No C=O O-H 3170-3427	# 9.817 15.46	m/e 344, 303, 256, 228	$\delta$ 4.51 5 $\beta$ H $\delta$ 3.51-3.55- 6 $\alpha$ H

<sup>&</sup>Chatterjee et al, 1975; \* tlc done on Alumina plates; # Different Mobile Phase from previous analyses; \*\* analysis was not performed.

Note - tlc = thin layer chromatography, IR = infra-red spectroscopy, HPLC = high pressure liquid chromatography, MS = mass spectrometry, NMR = nuclear magnetic resonance. See text for analysis conditions.

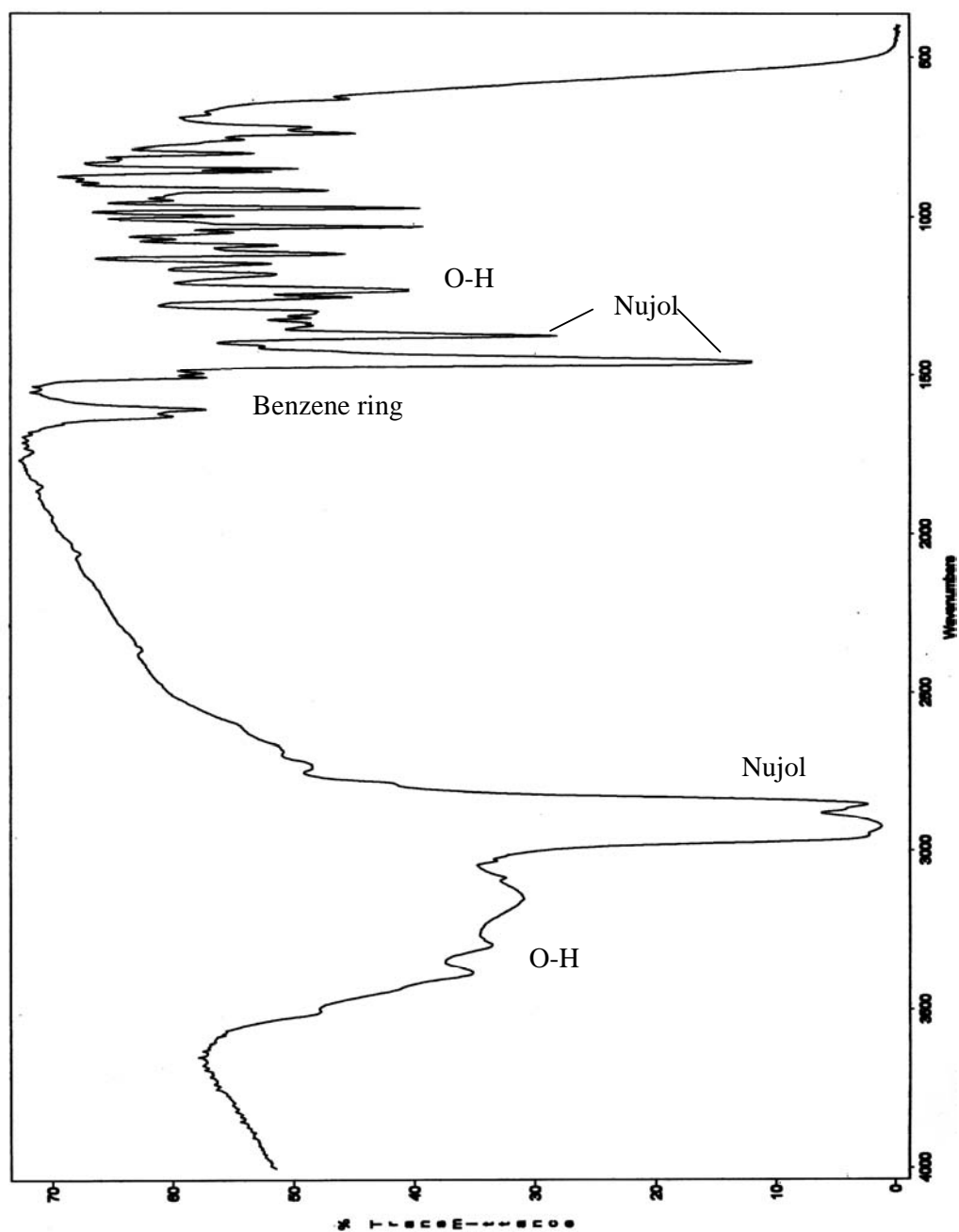


Figure 2.2 Infra-red spectroscopy of product 1.

Note absence of C=O at 1710 cm<sup>-1</sup>

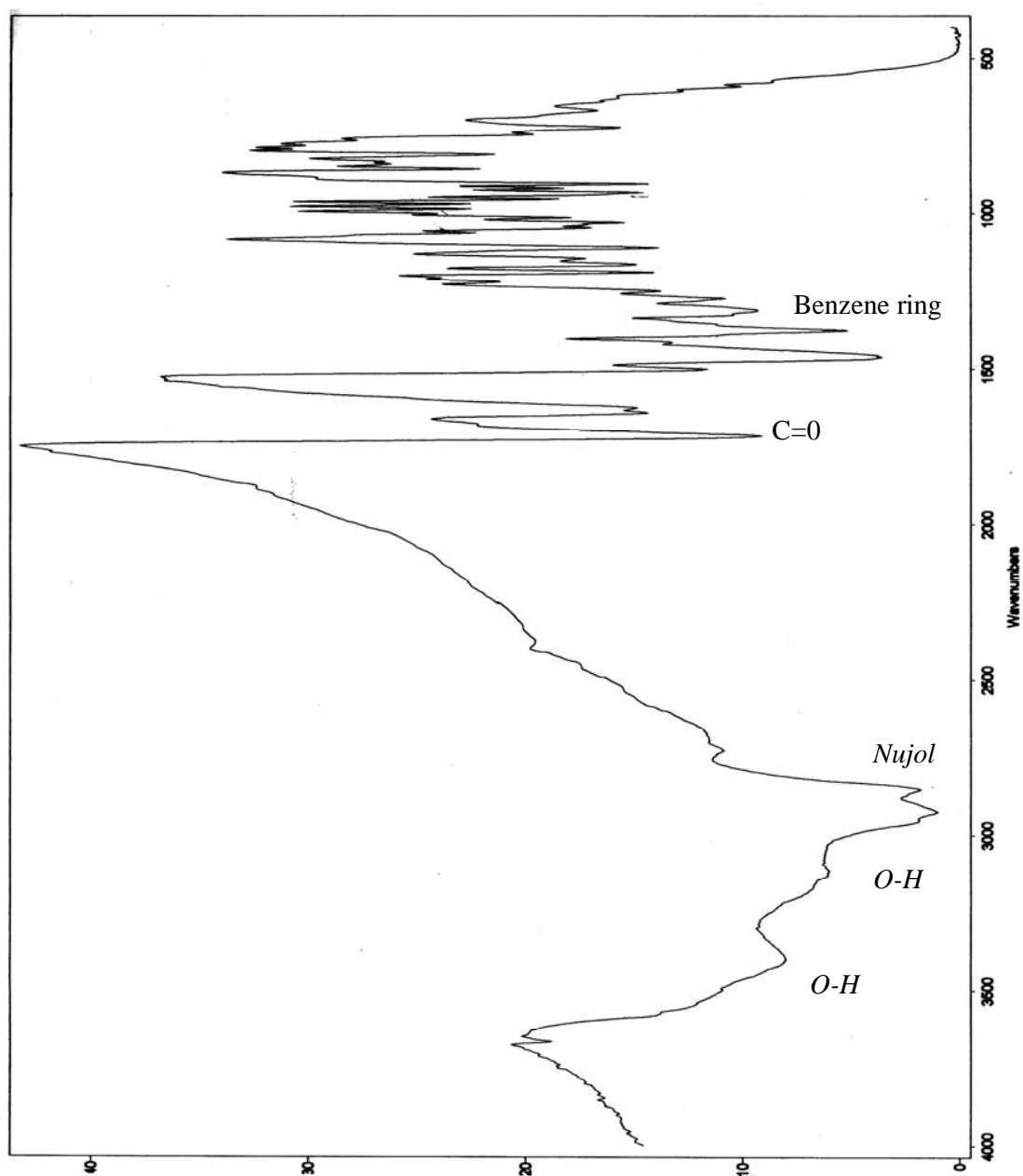


Figure 2.3 Infra-red spectroscopy of naltrexone hydrochloride.



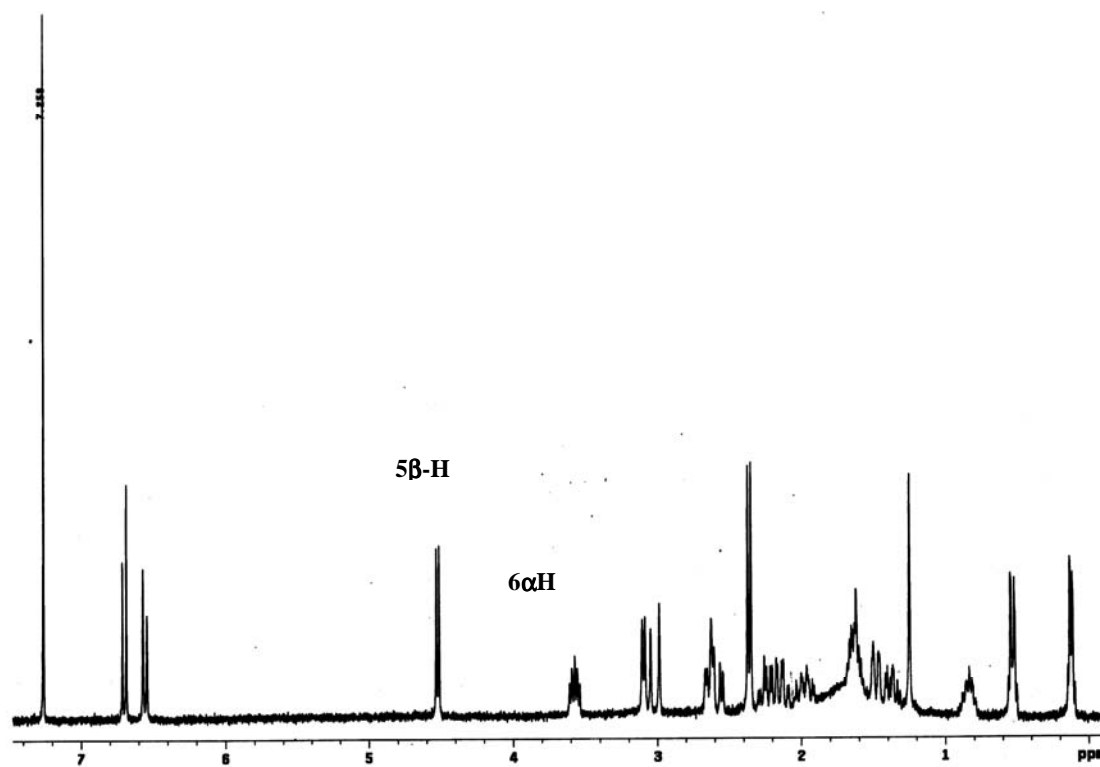


Figure 2.4 <sup>1</sup>H NMR of Product 1

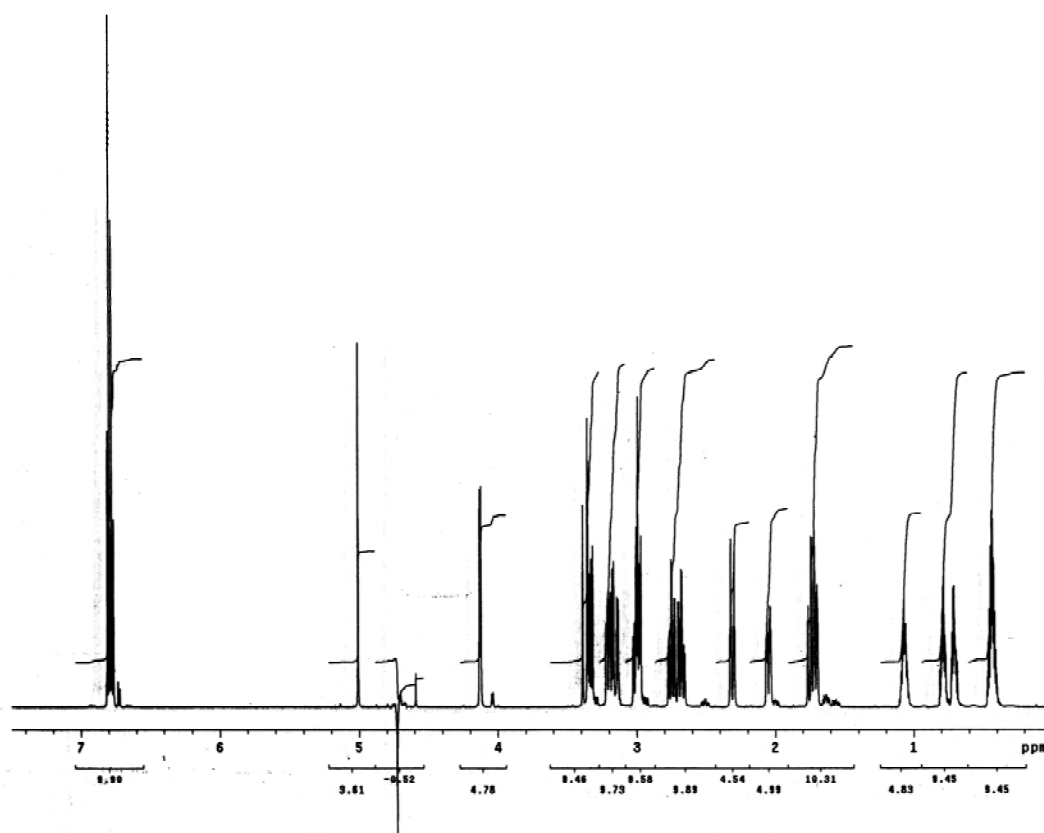


Figure 2.5 <sup>1</sup>H NMR of naltrexone hydrochloride

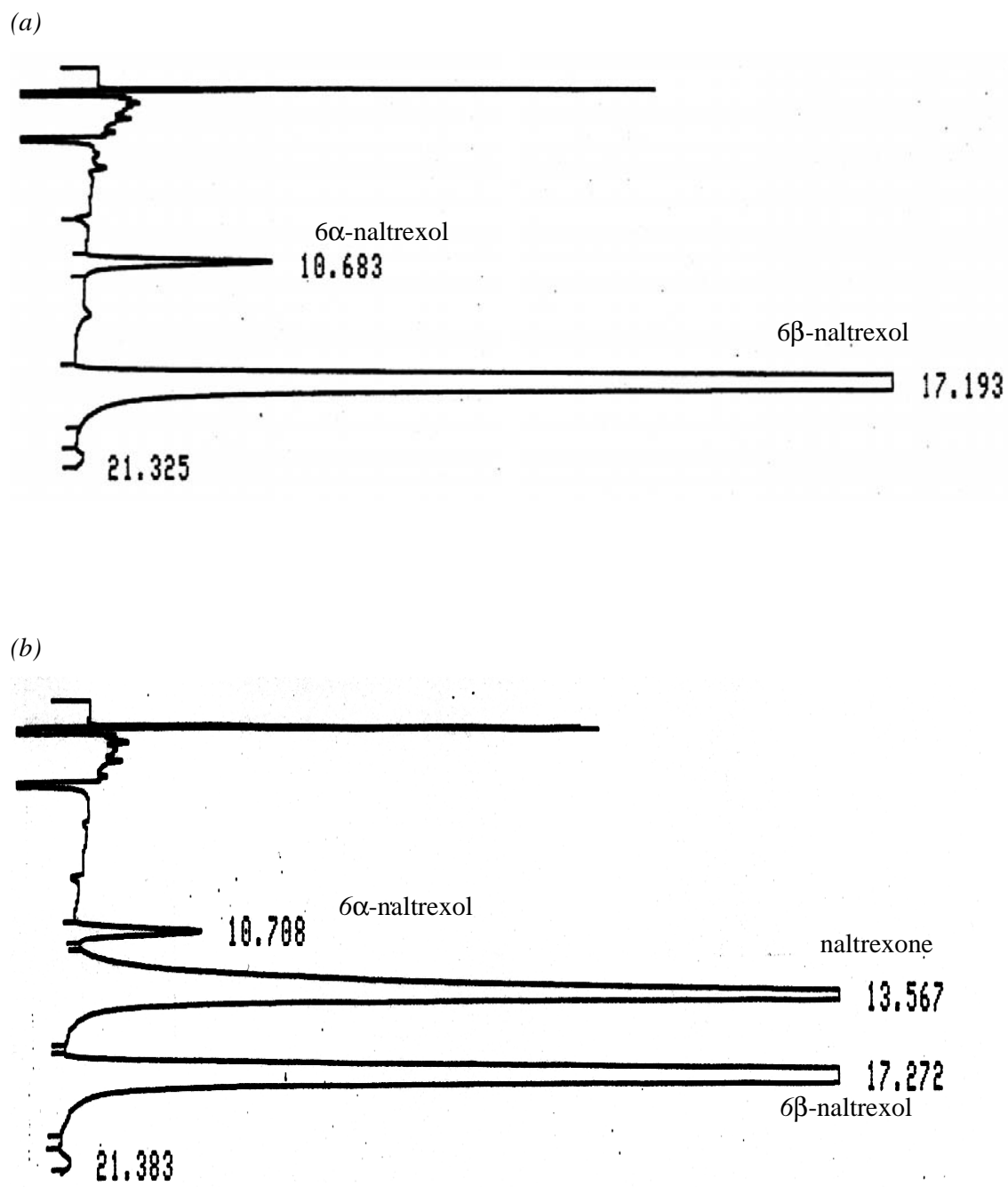


Figure 2.6 HPLC of (a) Product 1 ( $6\beta$ -naltrexol 20  $\mu\text{g/ml}$ ); (b) Product 1 ( $6\beta$ -naltrexol 20  $\mu\text{g/ml}$ ) and naltrexone (20  $\mu\text{g/ml}$ )

**2.4.2. Octanol:Phosphate buffer partitioning**

The octanol:pH 7.4 phosphate partitioning data are shown in Table 2.3. The mean ( $\pm$ SD) ratios obtained were 10.9 (2.2) for naltrexone, 26.4 (3.7) for naloxone, 3.1 (1.5) for 6 $\beta$ -naltrexol and 0.56 (0.17) for morphine.

Table 2.3 Octanol:pH 7.4 phosphate buffer partition coefficients of naltrexone, naloxone, 6 $\beta$ -naltrexol and morphine.

Compound	Partition coefficients (D)
Naltrexone 10 $\mu$ g/ml	13.50
50 $\mu$ g/ml	9.58
100 $\mu$ g/ml	9.72
Mean $\pm$ SD	10.9 $\pm$ 2.2
Naloxone 10 $\mu$ g/ml	30.7
50 $\mu$ g/ml	24.5
100 $\mu$ g/ml	24.0
Mean $\pm$ SD	26.4 $\pm$ 3.7
6 $\beta$ -Naltrexol 10 $\mu$ g/ml	4.83
50 $\mu$ g/ml	2.29
100 $\mu$ g/ml	2.11
Mean $\pm$ SD	3.1 $\pm$ 1.5
Morphine 10 $\mu$ g/ml	0.70
50 $\mu$ g/ml	0.38
100 $\mu$ g/ml	0.61
Mean $\pm$ SD	0.56 $\pm$ 0.17

Using these means, and literature pKa values for the proton on the nitrogen (Kaufmann *et al*, 1975a), the log P, the intrinsic partition coefficient of each compound was calculated. The log P of naltrexone, naloxone and 6 $\beta$ -naltrexol were calculated using the equation

$$\log D_{bases} = \log P + \log \left[ \frac{1}{(1 + 10^{pKa-pH})} \right]$$

(from Scherrer and Howard (1977)), where D is the distribution coefficient from the data  
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above. The pK<sub>a</sub> for 6 $\beta$ -naltrexol was taken to be the same as for naltrexone (at 37 °C). The pH used was 7.4. The resulting log P values were 1.982, 1.963 and 1.442 for naloxone, naltrexone and 6 $\beta$ -naltrexol, respectively.

## 2.5. Discussion

While Products 1 and 2 were different in colour their analyses proved to be the same, as shown in Table 2.1. The IR, NMR and MS results obtained for these products were consistent with those of the crude product obtained by Chatterjee *et al* (1975), with the exception that the products obtained in the current study were only 97% pure as shown by the HPLC, with approximately 3% 6 $\alpha$ -naltrexol present. The presence of the 6 $\alpha$ -naltrexol could account for the slight differences in shift on the proton NMR between the two studies. Products 3 and 4 were contaminated with both starting material, naltrexone, and thiourea dioxide, and were not used in any further experiments.

The mobile phase for the HPLC analyses of Products 5 and 6 was different from that previously used, thus accounting for the differences in retention time (R<sub>t</sub>) on the column shown in Table 2.2, and in all respects these compounds resemble Products 1 and 2, barring the presence of the extra hydrogen in the mass spectrometries.

In summary, the reduction of naltrexone hydrochloride using thiourea dioxide in an alkaline medium was successful, and sufficient yields of products 1 and 2 were obtained to conduct pharmacological experiments. The purity of the compounds was sufficient to perform the bioanalyses described below.

The mean octanol:phosphate buffer partition coefficient of morphine (0.56) was similar to that of 0.63 reported by Van Crugten *et al* (1991). As shown previously by Kaufmann *et al* (1975), naloxone had a much higher octanol:phosphate ratio than naltrexone, and higher than would be expected for the similarity in structure. The partition coefficient values obtained in the current study were significantly lower than those obtained by Kaufmann *et al* (1975) but they conducted their study at the pH corresponding to the pK<sub>a</sub> (of the nitrogen deprotonation) of the substrates, which are all higher than pH 7.4, at which the current study was conducted. The partition coefficient obtained in the current study for 6 $\beta$ -naltrexol was lower than that for either naltrexone or naloxone, indicating much lower lipophilicity, suggesting that the 6 $\beta$ -naltrexol would distribute more slowly across biological membranes such as the blood-brain barrier. This in turn would indicate that 6 $\beta$ -naltrexol would have a slower onset of action, but

potentially longer duration of action than either naloxone or naltrexone.

The retrospectively calculated log P values obtained are higher than those quoted by DrugBank for naltrexone (experimental 0.7, predicted 2.07), but similar to calculated log P for naltrexone of 1.80 by Berry *et al* (2009) and 1.608 (jlogP applet, v 1.2, JME Molecular Edition). This software calculated the log P for 6 $\beta$ -naltrexol to be 1.831. In agreement with Kaufmann *et al* (1975a) the absolute value of the distribution coefficient is a more sensitive measure in this instance of differences in lipophilicity than the calculated log P values.

### **3. High performance liquid chromatography (HPLC) quantification of naltrexone and 6 $\beta$ -naltrexol in biological fluids**

#### **3.1. Introduction**

There had been a number of published methods for the quantification of naltrexone and/or 6 $\beta$ -naltrexol in biological fluids when the current study was commenced in late 1997. Many of these have involved substantial sample work-up, consisting of complicated extraction and derivatisation procedures, the use of radiolabelled compounds and specialised equipment such as GC/MS, GC/MS/MS and liquid scintillation counters (see 1.8 of this thesis for review). In addition, 6 $\beta$ -naltrexol formation has been assessed in human liver cytosol preparations by measuring the oxidation rate of NADPH at 340 nm, with identification of products by thin layer chromatography (TLC) (Ohara et al., 1995), but prior to the current study, this metabolite had not been quantified in this preparation.

Davidson and colleagues in 1996 published a method for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in human plasma and urine, using simple organic and acid extraction procedures and HPLC with electrochemical detection (Davidson et al., 1996). The method presented in the current thesis is based on that by Davidson. The assay conditions for that method comprised an isocratic pump, a Waters 712 autosampler and a Coulochem II 5200 electrochemical detector. A Model 5020 guard cell was operated at a cell potential of +0.7 V, with an ESA Model 5011 high-sensitivity analytical cell with dual porous graphite working electrodes operated at two cell potentials. The first electrode was at +0.3 V and the second, where analyte detection occurred, at +0.65V. The mobile phase was methanol-phosphoric acid (50 mM) (20:80 v/v) at pH 3.2. The flow-rate was 1.2 ml/min, and analyte separation was achieved using a 100x4.6 mm i.d. (3  $\mu$ m) phenyl column. Nalbuphine was used as the internal standard for the extraction procedure. The specificity of the assay was determined by assaying drug-free control plasma from several different human donors to ensure that no endogenous chromatographic interferences were observed. The limits of quantification for this assay were 0.25 and 0.5 ng/ml for naltrexone and 6 $\beta$ -naltrexol, respectively, using 2 ml (a relatively large volume when collecting multiple samples) of plasma. The intra- and inter-day precision at five concentrations of naltrexone (0.25, 0.5, 5.0, 15 and 50 ng/ml) and 6 $\beta$ -naltrexol (0.5, 1.0, 10, 50 and 100 ng/ml) ranged from 0.5-7.7% and 0.7-8.2% respectively, for the two compounds.

#### **3.2. Specific aims of the current project**

The first aim of the current section was to develop and validate an HPLC method for the

quantification of 6 $\beta$ -naltrexol formed from the metabolism of naltrexone in human liver cytosol. Secondly, HPLC assays for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in biological fluids such as plasma, urine and saliva were developed and validated.

### 3.3. Chemicals

Naltrexone HCl, naloxone HCl, morphine HCl, oxycodone HCl, hydromorphone HCl, warfarin sodium bisulphate, bovine serum albumin (fraction V), Folin-Ciocalteu reagent, DL-isocitric acid tri-sodium salt, isocitrate dehydrogenase,  $\beta$ -nicotinamide adenine dinucleotide phosphate ( $\beta$ -NADPH reduced form) and NADP type IV were purchased from Sigma Aldrich (St Louis, Mo, USA). Racemic methadone was obtained from the National Institute on Drug Abuse (MD, USA), and oxymorphone HCl from Du Pont Pharmaceuticals (Wilmington, DE, USA). The 6 $\beta$ -naltrexol was synthesised as the free base in the Department of Chemistry, University of Adelaide by the method of Chatterjee *et al.*, (1975) (see Chapter 2). Methanol, acetonitrile and dichloromethane were of HPLC grade (BDH Laboratory Supplies, Poole, UK). Other chemicals used were potassium dihydrogen orthophosphate ( $\text{KH}_2\text{PO}_4$ ), sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) and orthophosphoric acid ( $\text{H}_3\text{PO}_4$ ), purchased from Merck Pty Ltd (Kilsyth Australia); sodium hydrogen carbonate ( $\text{NaHCO}_3$ ) (BDH Laboratory Supplies, Poole, UK), and triethylamine (TEA) (Prolabo, Paris, France).

Other chemicals used in the passivation of the Coulochem HPLC system were ethylenediamine tetra-acetic acid (EDTA) (BDH Chemicals, Kilsyth Australia) and glacial acetic acid (Ajax Chemicals, Sydney, Australia). All other chemicals and reagents were of analytical grade quality. Deionised analytical grade water was used for the preparation of all solutions.

### 3.4. Quantification of 6 $\beta$ -naltrexol in human liver cytosolic incubations

#### 3.4.1. Introduction

As previously mentioned, the formation of 6 $\beta$ -naltrexol from naltrexone has been studied by other investigators in human liver cytosol preparations. Ohara *et al.* (1995) examined the enzymes involved in this reaction, but measured the rate of formation of 6 $\beta$ -naltrexol by measuring the oxidation rate of NADPH rather than quantifying the metabolite *per se*. Prior to this, Malspeis and colleagues (1976) measured the *in vitro* reduction of naltrexone in cytosolic and microsomal fractions of guinea pig, monkey and rat liver preparations. The reduction products obtained from monkey and rat liver 9000 g supernatant preparations after

incubation of substrate (naltrexone) in the presence of an NADPH-generating system were analysed only qualitatively for 6 $\alpha$ - and 6 $\beta$ -naltrexol using TLC and GLC. Only the reaction products from the incubation of naltrexone with 9000 g and 105000 g supernatant fractions of guinea pig liver were quantified after extraction and derivatisation of samples using GC with electron capture detection.

### **3.4.2. HPLC instrumentation and chromatography conditions**

The formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosol was quantified using a reversed phase HPLC system comprising an LC-6A pump (Shimadzu Corporation, Kyoto, Japan) operating at a flow rate of 1.0 ml/min and a Wisp 710B autoinjector (Waters, Milford, MA, USA). The column was a Radial-Pak Cartridge packed with phenyl 5NVP 4  $\mu$  (Waters) which was inserted in a Radial Compression Module (RCM 8x10 mm) and operated at a pressure of approximately 2500 psi. A pre-column (Alltima C18 5  $\mu$ m, Alltech Corporation, IL, USA) was preceded by a 4  $\mu$ m inline filter.

The mobile phase was filtered through 0.2  $\mu$ m filters under vacuum and degassed by sonication prior to use. The mobile phase for optimal separation of analytes comprised 16% methanol, 0.2% TEA and 25 mM KH<sub>2</sub>PO<sub>4</sub> with the final pH adjusted to 3.0 with orthophosphoric acid. The mobile phase was degassed by filtration (Whatman glass fibre) and sonication prior to use. Detection of analytes was achieved using an ultraviolet detector (Jasco UVIDEK-100, Tokyo, Japan). A Shimadzu C-R6A Integrator (Shimadzu Corporation) was used for the integration of peak heights at 340 nm.

### **3.4.3. Cytosol assay method**

Cytosol was prepared as described below in Chapter 4.2. Retention times of the compounds of interest (naltrexone and 6 $\beta$ -naltrexol) were ascertained by direct injection of aqueous solutions of pure compounds. Quantification of 6 $\beta$ -naltrexol was performed with calibration curves consisting of eight standards in phosphate buffer (100 mM, pH 7.4) containing cytosolic protein (25  $\mu$ g) over the concentration range 0.25-20  $\mu$ M. The calibration samples were treated in the same manner as unknown and quality control samples. Cytosolic incubations of unknowns were performed in duplicate at 37°C in a shaking water bath (Julabo SW20-C, John Morris Scientific Pty, Ltd Sydney, Australia) for 60 minutes. The enzymatic formation of 6 $\beta$ -naltrexol was terminated by the addition of ice-cold methanol (100  $\mu$ l). This also served to precipitate any protein in the sample. The samples were then vortexed briefly,



centrifuged for 10 min at 14000 rpm, and 100  $\mu$ l of the supernatant was injected onto the HPLC system. No internal standard was used in this assay.

#### **3.4.4. Cytosol assay validation**

Inter- and intra-assay precision were monitored with quality control (QC) samples prepared in duplicate at three concentrations: low (LQC 1.75  $\mu$ M), medium (MQC 7.5  $\mu$ M) and high (HQC 14  $\mu$ M) and were also assayed in phosphate buffer (100 mM, pH 7.4) containing 25  $\mu$ g of cytosolic protein. The quality control samples were prepared from stock solutions (10x concentration) independently of calibration samples by a separate weighing (Mr Andrew Menelaou, Research Officer, University of Adelaide, Department of Clinical and Experimental Pharmacology). The lower limit of quantification (LOQ) was determined by analysis of the lowest calibration standard (n=6) for each analyte in a separate assay. LOQ was determined as the lowest calibration standard that produced a precision of < 20% and an inaccuracy of <  $\pm$  20%.

Samples containing phosphate buffer, NADPH and cytosolic protein alone (supernatants of methanol-precipitated samples) were injected onto the HPLC system to ascertain that there were no compounds co-eluting with the substrate and metabolite of interest.

#### **3.4.5. Cytosol assay data analysis**

Peak height data were entered into Excel spreadsheets (Version 4.0 Microsoft Corporation, WA, USA). Least-squares linear regression analysis (Regression, Blackwell Scientific Publications, Oxford UK) of peak height (weighted 1/y) against nominal concentration provided an estimate of slope, intercept and coefficient of determination ( $r^2$ ). The estimated concentration divided by the nominal concentration multiplied by 100% was determined for each calibration curve and QC sample and accuracy was determined as the mean of these values, and the residual standard deviation of the mean was taken as the precision.

#### **3.4.6. Cytosol assay results and discussion**

Retention times for naltrexone and 6 $\beta$ -naltrexol were 13 and 16 minutes, respectively. No component of the incubation buffer (phosphate buffer, NADPH and cytosolic protein) co-eluted with the substrate (naltrexone), or the formation product (6 $\beta$ -naltrexol). Figure 3.1 shows a chromatogram of a cytosolic incubation with naltrexone as the substrate. Linear regressions of the calibration curves (n=8) with weighting 1/y gave mean slope values of 5271 (CV 7.5%), and a mean correlation coefficient ( $r^2$ ) of 0.998 (CV 1.0%) as shown in Table 3.2.

The above assay was linear over the quantified concentration range and no compounds used in the inhibitor studies interfered with the quantification of 6β-naltrexol in this system. The mean inter-assay precision and accuracy values of the calibration curve samples (Table 3.1) and the mean intra- and inter-assay precision and accuracy of the quality control samples (Table 3.3) are given below. At the limit of quantification (0.25 μM), precision (n=20) was 8.6%, with a mean absolute accuracy of 88% (Table 3.3).

Based on the assay validation results shown in Table 3.1, assay acceptance criteria were defined as: calibration curve  $r^2$  values greater than 0.99; 6/8 standard values and 4/6 QC values less than 20% deviation from their nominal concentration.

The ongoing performance for the assay was monitored using the above criteria for the standard and quality control samples. These data are summarised in Chapter 4 (Table 4.3).

Table 3.1: Inter-assay accuracy and precision of the calibration curve samples for the quantification of 6β-naltrexol in human liver cytosol. (n=8 for all concentrations).

Nominal Concentration (μM)	Accuracy % (SD)	Precision %
0.25	93.5 (8.0)	9.1
0.5	103.5 (4.1)	5.4
1.0	111.9 (7.6)	6.9
2.5	91.8 (9.8)	13.2
5.0	95.4 (3.1)	3.3
10	97.0 (2.6)	4.1
15	102.2 (1.0)	2.4
20	101.9 (1.6)	2.4

Table 3.2. Mean precision and accuracy of slope values for the linear regression of the calibration curves of 6β-naltrexol quantification in human liver cytosol.

n	Regression coefficient ( $r^2$ )	Regression coefficient Precision (%)	Mean Slope	Slope (%) CV
8	0.9982	1.0	5271	7.49

Table 3.3: Inter- and intra-assay accuracy and precision for the quantification of 6 $\beta$ -naltrexol in human liver cytosol. LOQ is the limit of quantification of the assay, LQC, MQC and HQC are the low, mid and high quality controls, respectively.

	n	Nominal Conc. ( $\mu$ M)	Accuracy % (SD)	CV %
<b>Inter-assay</b>				
LQC	16	1.75	89.9 (12.9)	16.2
MQC	14	7.5	97.6 (0.9)	2.63
HQC	16	14	95.8 (2.6)	3.10
<b>Intra-assay</b>				
LOQ	20	0.25	87.8 (5.0)	8.6
LQC	10	1.75	91.9 (14.1)	14.4
MQC	10	7.5	97.1 (1.2)	1.16
HQC	10	14.0	94.1 (1.2)	1.23

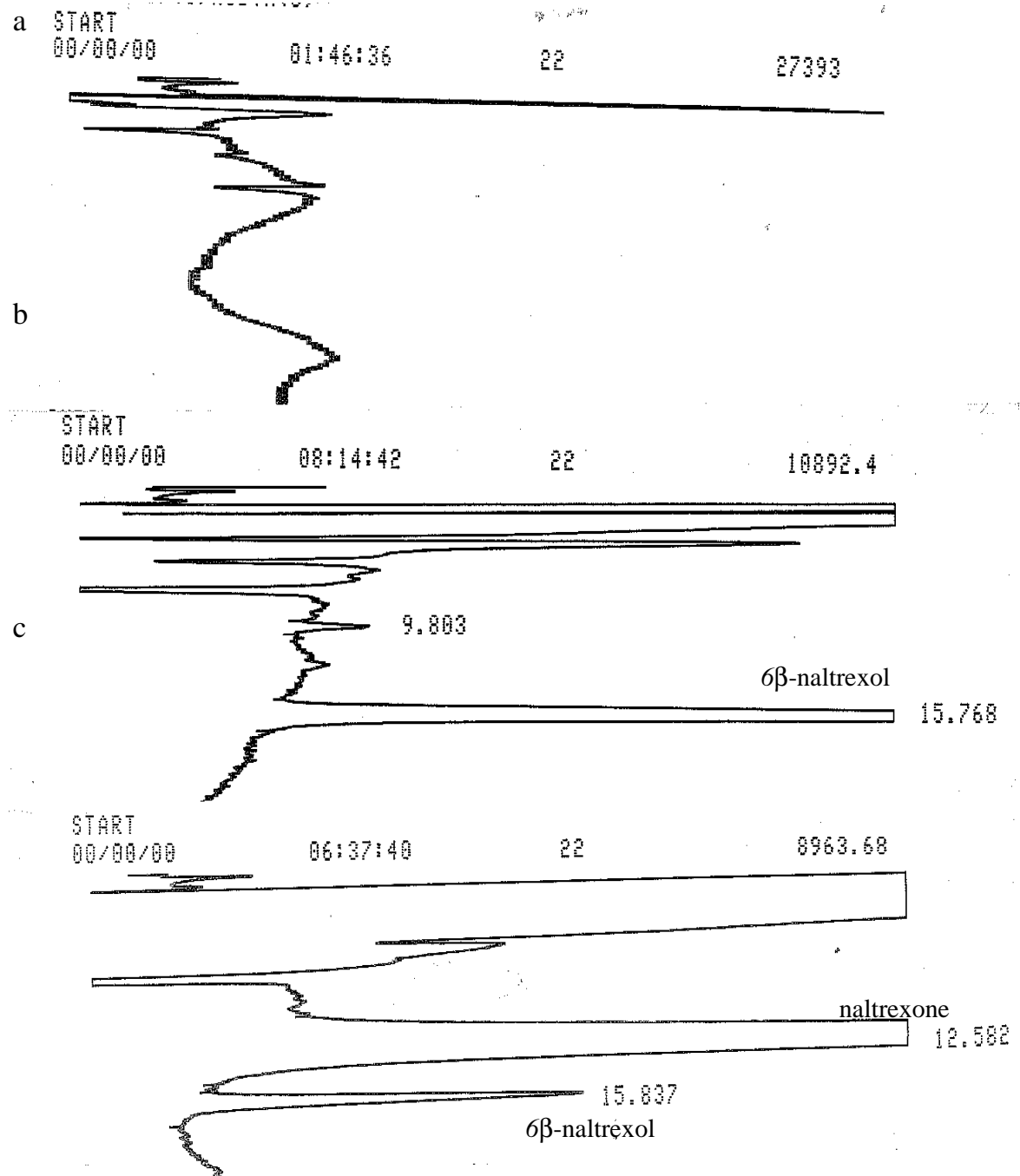


Figure 3.1 HPLC of 6 $\beta$ -naltrexol formation from naltrexone in human liver cytosol. Chromatogram a) blank cytosol; chromatogram b) low (1.75  $\mu$ M) 6 $\beta$ -naltrexol quality control; chromatogram c) formation of 6 $\beta$ -naltrexol (15.8 min) in cytosol (0.36  $\mu$ M), using naltrexone (12.6 min) as the substrate.

### 3.5. Quantification of naltrexone and 6 $\beta$ -naltrexol in plasma

#### 3.5.1. Introduction

Several methods have been published for the quantification of naltrexone and 6 $\beta$ -naltrexol in plasma, but few have been simultaneous analyses and most have involved extended work-up of the samples. The method for the simultaneous quantification of plasma naltrexone and 6 $\beta$ -naltrexol concentrations used in the present thesis was a modification of that of Davidson *et al* (1996).

#### 3.5.2. Plasma assay conditions

The assay conditions used for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol consisted of a reversed phase HPLC system with electrochemical detection (ECD). The system comprised an LC-6A pump (Shimadzu, Kyoto, Japan) and a Shimadzu Sil 9A autoinjector (Shimadzu, Kyoto, Japan). The column was a Radial-Pak Cartridge packed with phenyl 5NVPH 4 $\mu$  (Waters) which was inserted in a Radial Compression Module (RCM 8x10 mm), operated at a pressure of approximately 2500 psi. No pre-column was used. The detector was a Coulochem II 5200 electrochemical detector (ESA, Bedford, MA, USA). A Model 5020 guard cell (ESA) operated at a cell potential of +0.7 V was used to oxidise impurities from the mobile phase and to reduce system noise and background current. An ESA Model 5010 analytical cell with dual porous graphite working electrodes was operated at two cell potentials. The first electrode was set at +0.30 V to eliminate oxidation of endogenous components of plasma and urine extracts at the second analyte detection electrode, operated at 0.65 V. Peak heights were integrated using a Shimadzu C-R6A Integrator (Shimadzu Corporation). The range of the detector was set at 200 an.

The mobile phase used for the optimal separation of naloxone (internal standard), naltrexone and 6 $\beta$ -naltrexol comprised 16% methanol in 50 mM KH<sub>2</sub>PO<sub>4</sub> adjusted to a pH of 3.2 with orthophosphoric acid. The mobile phase was filtered through 0.2  $\mu$ m filters under vacuum and degassed by sonication. The flow rate of the mobile phase was 0.8 ml/min. The above conditions resulted in retention times of 9, 14 and 18 minutes for naloxone, naltrexone and 6 $\beta$ -naltrexol, respectively.

#### 3.5.3. Plasma sample preparation

Drug-free human plasma samples (1.0 ml) plus internal standard (100  $\mu$ l of 100 ng/ml [in water] naloxone) were treated with 1.0 ml acetonitrile in order to precipitate the plasma

proteins. This was necessary to obtain a recovery of 6β-naltrexol greater than 80%. The samples were then stored at 4 °C overnight. After centrifuging for 10 min at 3500 rpm, the samples were transferred to new 10 ml flat-bottomed plastic tubes, alkalinised with 500 µl 1 M Na<sub>2</sub>CO<sub>3</sub> (pH 9.0), and extracted with 5 ml dichloromethane for 30 min on a rotary mixer. After the samples were centrifuged for 10 min at 3500 rpm, the upper aqueous layer was removed and the samples were washed with an additional 500 µl Na<sub>2</sub>CO<sub>3</sub> by vortexing for 30 sec. After centrifugation (10 min, 3500 rpm), the aqueous layer was again removed, and the organic layer was transferred to a new 10 ml tube containing 200 µl of 50 mM KH<sub>2</sub>PO<sub>4</sub> (pH 2.0). The samples were then mixed for 30 min on a rotary mixer, centrifuged for 10 min at 3500 rpm and 170 µl of the acid bubble was transferred to injection vials.

#### **3.5.4. Plasma assay validation procedure**

Retention times of the compounds of interest were confirmed by direct injection of aqueous solutions of pure compounds. Quantification of naltrexone and 6β-naltrexol from unknown and quality control samples was performed with calibration curves consisting of eight standards over the concentration range 0.5-25 ng/ml (naltrexone) and 2-100 ng/ml (6β-naltrexol) as the free base. Initially, 6β-naltrexol standards were in the range 0-25 ng/ml, and intra-assay and LOQ validations were performed with this calibration curve. Subsequently, the 6β-naltrexol calibration curve samples were increased to the range of 2-100 ng/ml. All plasma calibration and QC samples were prepared as 10-fold concentrated stocks and diluted on the day of assay in drug-free plasma. Subjects' samples which contained naltrexone or 6β-naltrexol at concentrations above the analytical limit of quantification were diluted in drug-free plasma and re-extracted.

Naltrexone and 6β-naltrexol inter- and intra-assay precision and accuracy were monitored with quality control (QC) samples prepared as previously described in duplicate at three concentrations: low (LQC 1.75 and 7.5 ng/ml), medium (MQC 7.5 and 37.5 ng/ml) and high (HQC 17.5 and 75 ng/ml) for the two compounds, respectively. The lower limit of quantification (LOQ) was determined by analysis of the lowest calibration standard (n=6) for each analyte in a separate assay. LOQ was determined as the lowest calibration standard that produced a precision of < 20% and an inaccuracy of < ± 20%.

The extraction recoveries of the compounds of interest (naltrexone, naloxone and 6β-naltrexol) were assessed by comparison of the peak heights obtained in an aqueous solution

and post-extraction of the quality control samples.

The specificity of the assay was assessed by injecting extracted drug-free plasma samples. All drug-free plasma used in the dilution of calibration standards and QC samples and baseline samples from the volunteers in the pharmacokinetic study (see Chapter 5) were analysed to ensure there were no compounds co-eluting with the compounds of interest.

### 3.5.5. Calibration curve and validation analysis

Integrated peak height data were entered into Excel spreadsheets (Version 4.0 Microsoft Corporation, WA, USA). Peak heights were converted into peak height ratios using the peak height of the internal standard (naloxone). Least-squares linear regression analysis (Regression, Blackwell Scientific Publications, Oxford UK) of peak height ratio (weighted 1/y) against nominal concentration provided an estimate of slope, intercept and coefficient of determination ( $r^2$ ). The estimated concentration divided by the nominal concentration multiplied by 100% was calculated for each individual sample and accuracy was calculated as the mean of these values, while the residual standard deviation of the mean was taken as the precision.

### 3.5.6. Results and Discussion

Injection of aqueous samples of pure compounds resulted in retention times of 9, 14 and 18 minutes for naloxone, naltrexone and 6 $\beta$ -naltrexol, respectively. Drug-free plasma samples exhibited no peaks corresponding to the compounds of interest. Figure 3.2 shows representative chromatograms of plasma containing naltrexone, 6 $\beta$ -naltrexol and the internal standard naloxone.

Davidson *et al* (1996) reported extraction recoveries of 95 $\pm$ 13.6% and 74 $\pm$ 5.2% for naltrexone and 6 $\beta$ -naltrexol respectively at pH 7.0, but in the current study the recovery for 6 $\beta$ -naltrexol more closely resembled that of Meyer *et al* (1984), of 45% (Table 3.4A), therefore, a precipitation step was required, which increased the recovery to >80% (Table 3.4B). The low recovery of 6 $\beta$ -naltrexol was improved by changing the pH of the extraction from 7.0 to 9.0. Ethanol and acetone were also used to precipitate the plasma proteins prior to extraction, and both improved recovery, but not to the same extent as acetonitrile which was used in all assays. It is not known why there was such a difference in extraction recoveries between naltrexone and 6 $\beta$ -naltrexol in the plasma in the current assay compared to that of Davidson *et al* (1996).

Linear regression analyses of the calibration curves (n=8) with weighting 1/y gave mean slope values of 0.061 (CV 6.5%), and a mean correlation coefficient ( $r^2$ ) of 0.9987 (CV 0.08%) for naltrexone and 0.071 (CV 5.2%) and 0.9978 (CV 0.15%) for mean 6 $\beta$ -naltrexol slope and correlation coefficient (Table 3.5).

Mean inter- and intra-assay precision and accuracy values are summarised in Table 3.6. At all QC concentrations for both naltrexone and 6 $\beta$ -naltrexol, the imprecision was less than 10%, with an accuracy of 90% or above. At the limit of quantification (0.5 ng/ml naltrexone, 2.0 ng/ml 6 $\beta$ -naltrexol), precision (n=6) was 2.4%, with a mean absolute accuracy of 93.6% for naltrexone and 3.8% and 94% precision and accuracy respectively, for 6 $\beta$ -naltrexol (Table 3.6).

Based on the assay validation results above, assay acceptance criteria for all further assays were defined as: calibration curve  $r^2$  values greater than 0.99; less than 20% deviation from the nominal concentration for at least 6 of the 8 standard values and 4 of the 6 QC values. The ongoing performance of the plasma assay was monitored using the above criteria, and these data are summarised in section 5.1. The above assay was linear over the quantified concentration ranges and no endogenous compounds interfered with the quantification of naltrexone or 6 $\beta$ -naltrexol in this system.

Table 3.4. Extraction efficiencies for naloxone, naltrexone and 6 $\beta$ -naltrexol from plasma (A) before (n=6) and (B) after (n=11) acetonitrile precipitation of the proteins in plasma. LQC=low quality control; MQC=medium quality control; HQC=high quality control.

Sample	Naloxone Mean % Recovery (SD)		Naltrexone Mean % Recovery (SD)		6 $\beta$ -Naltrexol Mean % Recovery (SD)	
	A	B	A	B	A	B
LQC	81.3 (19.5)	90.5 (0.02)	71.3 (15.1)	83.2 (11.7)	25.6 (13.1)	76.7 (9.8)
MQC	78.1 (6.1)	78 (0)	77.4 (15.1)	80.9 (1.8)	21.7 (9.3)	73.3 (4.6)
HQC	87.4 (14.3)	100 (4.0)	86.3 (13.0)	100 (22.0)	23.9 (9.4)	92.2 (20.3)



Table 3.5. Mean Precision and accuracy of slope values for the linear regression of the calibration curves of 6 $\beta$ -naltrexol and naltrexone quantification in human plasma.

	n	Regression coefficient (r <sup>2</sup> )	Regression coefficient Precision (%)	Mean	% (CV)
Naltrexone	8	0.9987	0.08	0.0608	6.5
6 $\beta$ -naltrexol	8	0.9978	0.15	0.0709	5.2

Table 3.6: Inter- and intra-assay accuracy and precision for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in human plasma. LOQ is the limit of quantification of the assay, LQC, MQC and HQC are low, mid and high quality controls, respectively.

	n	Naltrexone			6 $\beta$ -naltrexol		
		Conc. (ng/ml)	Accuracy %	Precision %	Conc. (ng/ml)	Accuracy %	Precision %
<b>Inter-assay</b>							
LOQ	6	0.5	93.6	2.4	2.0	94.0	3.8
LQC	16	1.75	97.8	5.38	17.5	100.8	7.16
MQC	14	7.5	90.5	2.32	37.5	95.9	3.74
HQC	16	17.5	93.8	1.82	75	98.4	3.75
<b>Intra-assay</b>							
LQC	8	1.75	96.4	6.10	1.75	103.5	1.11
MQC	8	7.5	89.6	3.57	7.5	97.8	0.68
HQC	8	17.5	93.3	0.82	17.5	97.1	1.01

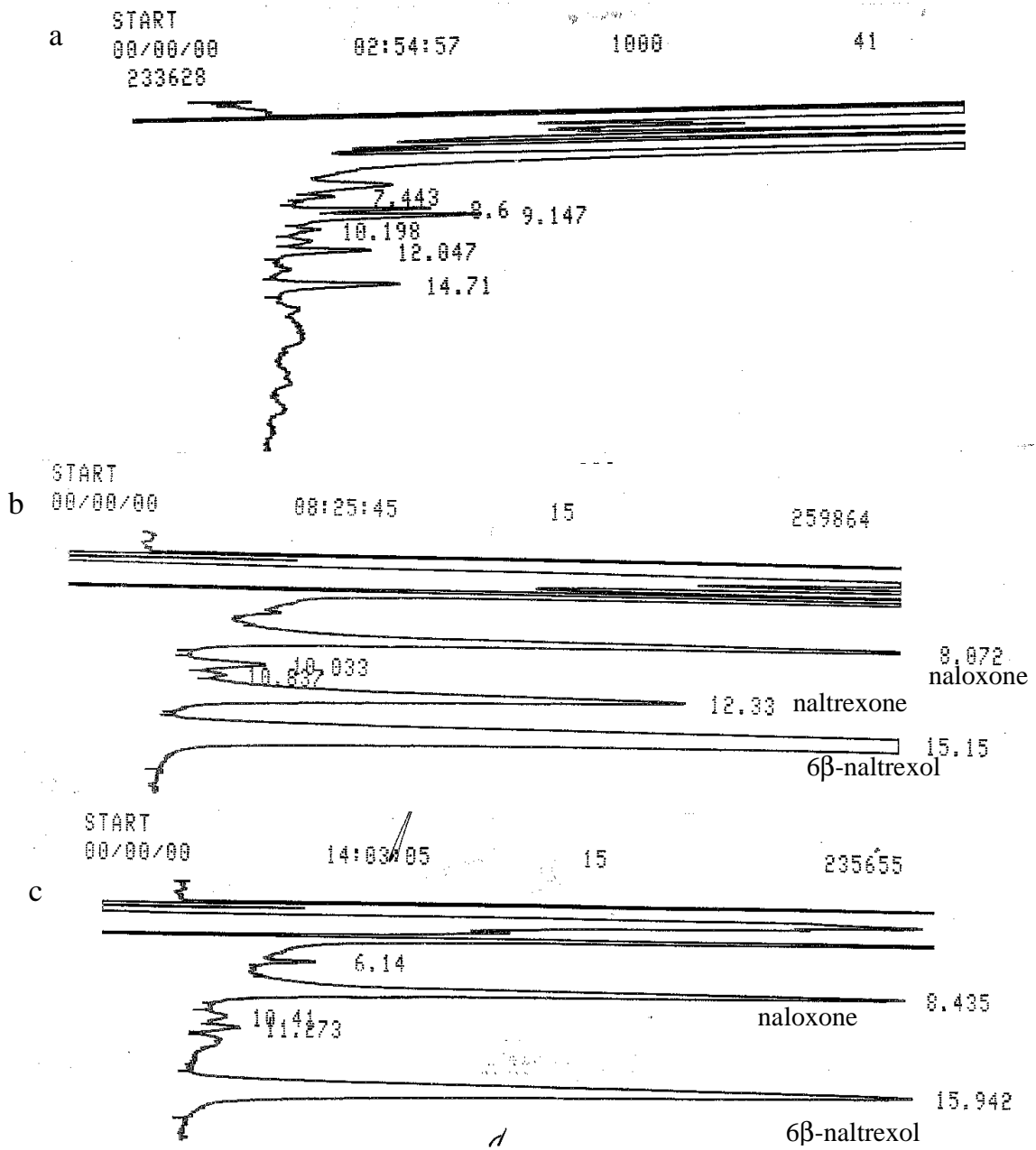


Figure 3.2 HPLC of human plasma.

Chromatogram a) blank plasma; chromatogram b) mid quality control (MQC), naloxone (internal standard 8.1 min), naltrexone 7.5 ng/ml (12.3 min) and 6 $\beta$ -naltrexol 37.5 ng/ml (15.2 min); chromatogram c) volunteer sample 30 hr post- 50 mg oral naltrexone, naltrexone not detected, 6 $\beta$ -naltrexol 10 ng/ml (15.9 min).

### 3.6. Simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in urine

#### 3.6.1. Urine assay conditions

The initial assay conditions used for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in urine were the same as those for plasma described above (3.4.1). Due to the high concentrations of naltrexone and 6 $\beta$ -naltrexol in some of the human subjects' urine samples (See 5.3), the conditions for analysis had to be modified. These modifications included constructing calibration curves in the range of 5-100 ng/ml for naltrexone, and 100-2000 ng/ml for 6 $\beta$ -naltrexol. When using the latter 6 $\beta$ -naltrexol calibration curve, the range of the detector was 5  $\mu$ A rather than 200 nA. Additionally, injection volumes for the HQC samples and the calibration standards of 60, 80 and 100 ng/ml (naltrexone), and 1500, 1750 and 2000 ng/ml (6 $\beta$ -naltrexol), were decreased to 30  $\mu$ l, instead of the usual 100  $\mu$ l injected. The mobile phase was as described for plasma, above (3.4.1). The flow rate of the mobile phase was also the same at 0.8 ml/min.

#### 3.6.2. Urine sample preparation

Urine samples spiked with known concentrations of analytes (naltrexone and 6 $\beta$ -naltrexol) of 100  $\mu$ l were added to 900  $\mu$ l drug-free urine containing internal standard (100  $\mu$ l of 100 ng/ml naloxone). The urine samples were alkalised with 500  $\mu$ l of 1M Na<sub>2</sub>CO<sub>3</sub> (pH 9.0) and extracted with 5 ml dichloromethane for 30 min on a rotary mixer. After centrifuging the samples for 10 min at 3500 rpm, the upper aqueous layer was removed and the samples were washed with an additional 500  $\mu$ l of the Na<sub>2</sub>CO<sub>3</sub> buffer by vortexing for 30 sec. After centrifugation (10 min, 3500 rpm), the aqueous layer was again removed and the organic layer was transferred to a new 10 ml tube containing 200  $\mu$ l of 50 mM KH<sub>2</sub>PO<sub>4</sub> (pH 2.0). The samples were then mixed for 30 min on a rotary mixer, centrifuged for 10 min at 3500 rpm and 170  $\mu$ l of the acid bubble was transferred into injection vials.

#### 3.6.3. Urine assay validation

Initially, quantification of naltrexone and 6 $\beta$ -naltrexol was performed with calibration curves consisting of eight standards over the concentration range 0.5-25 ng/ml (naltrexone) and 2-100 ng/ml (6 $\beta$ -naltrexol). Inter- and intra-assay validations were performed with this calibration curve. In order to quantitate both naltrexone and 6 $\beta$ -naltrexol in glucuronidase-hydrolysed samples (to deconjugate from the glucuronide as described in 5.3.4.4), and 6 $\beta$ -naltrexol in some of the unhydrolysed samples, calibration curves of naltrexone (5, 10, 20, 40, 60, 80 and 100 ng/ml) and 6 $\beta$ -naltrexol (100, 250, 500, 750, 1000, 1500, 1750 and 2000

ng/ml) were constructed. These naltrexone and 6 $\beta$ -naltrexol assays were not performed simultaneously as the detector sensitivity required adjustment. A low volume validation was performed using quality control values of 15, 50 and 75 ng/ml for naltrexone and 350, 700 and 1250 ng/ml for 6 $\beta$ -naltrexol. All urine standards and QC samples were prepared as 10-fold concentrated stocks and diluted on the day of assay in drug-free urine.

Naltrexone and 6 $\beta$ -naltrexol inter- and intra-assay precision and accuracy were monitored with quality control (QC) samples prepared in duplicate at three concentrations: low (LQC 1.75 and 7.5 ng/ml), medium (MQC 7.5 and 37.5 ng/ml) and high (HQC 17.5 and 75 ng/ml) for naltrexone and 6 $\beta$ -naltrexol, respectively. The ongoing performance of the separate high concentration analyte assays was assessed using quality control (QC) samples with concentrations of LQC 15 ng/ml, MQC 50 ng/ml and HQC 75 ng/ml for naltrexone, and LQC 350 ng/ml, MQC 700 ng/ml and HQC 1250 ng/ml for 6 $\beta$ -naltrexol. The lower limit of quantification (LOQ) was determined by analysis of the lowest calibration standard (n=6) for each analyte in a separate assay. LOQ was determined as the lowest calibration standard that produced a precision of < 20% and an inaccuracy of <  $\pm$  20%.

The extraction recoveries of the compounds of interest (naltrexone, naloxone and 6 $\beta$ -naltrexol) were assessed by comparison of the peak heights obtained in an aqueous solution and post-extraction of the quality control samples.

The specificity of the assay was assessed by injecting extracted drug-free urine samples. All drug-free urine used in the dilution of calibration standards and QC samples and baseline samples from the volunteers in the kinetic study (see Chapter 5) were analysed to ensure there were no compounds co-eluting with the compounds of interest.

Based on the assay validation results, assay acceptance criteria were defined as: calibration curve  $r^2$  values greater than 0.99; 6/8 standard values and 4/6 QC values less than 20% deviation from their nominal concentration. The calculation of concentrations and analyses of precision and accuracy were performed as described in section 3.5.5 above.

#### **3.6.4. Urine assay results and discussion**

The above conditions resulted in retention times of 9, 14 and 18 min for naloxone, naltrexone and 6 $\beta$ -naltrexol, respectively. The drug-free urine samples (baseline volunteer samples and diluent for calibration standards and quality control samples) contained no components that

co-eluted with the peaks of interest to cause interference with the assay. Figure 3.3 shows the HPLC of a sample of urine in a volunteer following an oral dose (50 mg) of naltrexone, with the internal standard naloxone.

Linear regression analyses of the calibration curves (n=8) with weighting 1/y gave mean slope values of 0.074 and a mean correlation coefficient ( $r^2$ ) of 0.993 for naltrexone and 0.068 and 0.996 for mean 6β-naltrexol slope and correlation coefficient, respectively (Table 3.7). Mean inter- and intra-assay precision and accuracy values are summarised in Table 3.8.

The assay described above shows that naltrexone and 6β-naltrexol can be quantified simultaneously in human urine samples with acceptable precision and accuracy with a limit of quantification of 0.53 ng/ml (naltrexone and 6β-naltrexol). Due to the high metabolic conversion to the metabolite, and extensive conjugation of both the parent drug and the metabolite, the assay calibration curve and QC ranges had to be increased for the quantification of naltrexone and 6β-naltrexol in deconjugated samples.

Table 3.7. Mean Precision and accuracy of slope values for the linear regression of the calibration curves of 6β-naltrexol and naltrexone quantification in human urine.

	n	Regression coefficient ( $r^2$ )	Precision % CV	Mean Slope	% CV Slope
<b>Naltrexone</b>	8	0.9935	0.29	0.0783	20.0
<b>6β-naltrexol</b>	8	0.9957	0.20	0.0682	15.7

Table 3.8 Inter- and intra-assay accuracy and precision for the simultaneous quantification of naltrexone and 6β-naltrexol in human urine. LQC, MQC and HQC represent the low, medium and high quality controls, respectively.

	n	Naltrexone			6β-naltrexol		
		Conc. (ng/ml)	Accuracy %	Precision % CV	Conc. (ng/ml)	Accuracy %	Precision % CV
<b>Inter-assay</b>							
<b>LQC</b>	16	1.75	98.2	12.99	7.5	97.4	6.57
<b>MQC</b>	14	7.5	96.4	9.14	37.5	92.4	5.77
<b>HQC</b>	16	17.5	99.8	11.28	75	90.5	8.22
<b>Intra-assay</b>							
<b>LQC</b>	8	1.75	80.3	7.90	7.5	98.8	1.09
<b>MQC</b>	8	7.5	93.3	1.63	37.5	90.7	1.42
<b>HQC</b>	8	17.5	92.4	2.72	75	97.8	2.99

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Table 3.9 Intra-assay accuracy and precision of high concentration QC values for naltrexone and 6β-naltrexol in urine. Note: Assays not performed simultaneously. LQC, MQC and HQC represent the low, medium and high quality controls, respectively.

Urine	Naltrexone				6β-naltrexol			
	n	Conc. (ng/ml)	Accuracy %	Precision % CV	n	Conc. (ng/ml)	Accuracy %	Precision % CV
<b>Intra-Assay</b>								
<b>LQC</b>	2	15	97.6	0.29	2	350	98.6	1.53
<b>MQC</b>	2	50	94.2	1.32	2	700	98.7	0.61
<b>HQC</b>	2	75	95.0	0.46	2	1250	91.8	0.94

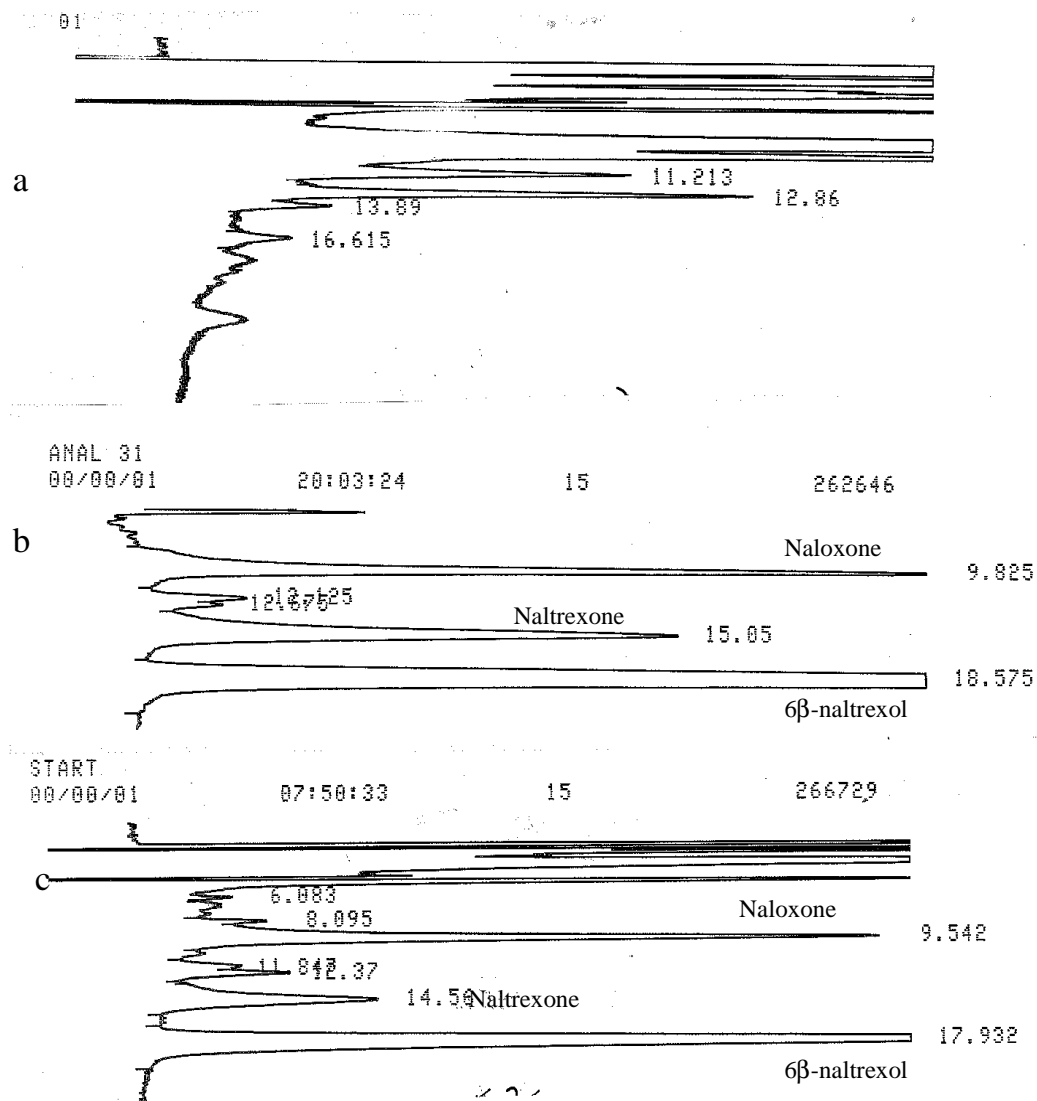


Figure 3.3 HPLC of human urine. Chromatogram a) blank urine; chromatogram b) mid quality control (MQC), naloxone (internal standard 9.8 min), naltrexone 7.5 ng/ml (15.1 min) and 6β-naltrexol 37.5 ng/ml (18.6 min); chromatogram c) volunteer sample 3-6 hr post-1 mg IV naltrexone, naltrexone 2.6 ng/ml (14.6 min), 6β-naltrexol 26 ng/ml (17.9 min).

### 3.7. Simultaneous assay for naltrexone and 6 $\beta$ -naltrexol in plasma ultrafiltrates

#### 3.7.1. Introduction

Plasma samples from the human pharmacokinetic study (See 5.5) were ultrafiltered in order to determine the extent of protein binding of both naltrexone and 6 $\beta$ -naltrexol in plasma. There have been few studies which have examined the plasma protein binding of naltrexone and 6 $\beta$ -naltrexol. Ludden *et al* (1976) demonstrated that equilibrium dialysis using tritiated naltrexone resulted in naltrexone binding to plasma proteins of  $20.3 \pm 0.47\%$  (n=3). This was independent of naltrexone concentration (range 0.1-500 ng/ml). However, as the metabolites of naltrexone were not differentiated from the parent compound and the radioactivity of the dialysed samples only was measured, it is not known if this value represents binding of naltrexone only, or naltrexone and metabolites, a point mentioned in the discussion of the study.

#### 3.7.2. Ultrafiltrate assay conditions

The assay conditions for the quantification of naltrexone and 6 $\beta$ -naltrexol in plasma ultrafiltrates were as described in section 3.5.2 above for plasma, with the exception that the calibration curve and quality control samples were diluted in phosphate-buffered saline (pH 7.4) rather than plasma.

#### 3.7.3. Ultrafiltrate sample preparation

The equipment used to obtain ultra-filtrates of the plasma comprised an ultrafiltration device (MPS-1, Amicon, MA USA) and a centrifuge (Beckman J2-21) with a fixed-angle rotor (Beckman JA-20.1). The devices were passivated prior to use as per the manufacturer's instructions by repeated washing with isotonic phosphate buffer and centrifugation. In order to determine the extent of non-specific binding of naltrexone and 6 $\beta$ -naltrexol to the ultrafiltration devices (MPS-1, Amicon, MA USA), aqueous samples of 5 and 50 ng/ml in phosphate-buffered saline (pH 7.4) were filtered through the membrane and the peak heights compared to peak heights of non-filtered solutions of the same concentrations. Drug-free plasma samples were also spiked with naltrexone and 6 $\beta$ -naltrexol (100 ng/ml) and filtered through the devices.

Plasma samples (1.0 ml) were adjusted to pH 7.4, with 5% orthophosphoric acid (H<sub>3</sub>PO<sub>4</sub>), placed in a pre-warmed sample reservoir and warmed to 37°C in a shaking water bath for 15min. The centrifuge chamber and fixed-angle rotor were also pre-warmed to 37°C by centrifugation at 16000 rpm for 15 min. The plasma was filtered by centrifugation for 20 min

*Susan J Porter, PhD Thesis, 2010*

at 3500 rpm (1500 g, compensation 0). A proportion (300  $\mu$ l) of the ultrafiltrate was transferred to 10 ml flat-bottomed tubes and extracted as per plasma samples described in 3.5.3. above, except that samples no longer required protein precipitation with acetonitrile. A control sample of 300  $\mu$ l of unfiltered plasma was extracted and assayed simultaneously.

#### **3.7.4. Ultrafiltrate assay validation**

The assay used for the quantification of naltrexone and 6 $\beta$ -naltrexol in the ultrafiltrates was the same as that for the plasma assay described above (3.5.3), with the exception of the altered dilution solution for standards and quality control samples; therefore, this assay was not revalidated. The acceptance criteria for the assay of unknown patient samples were the same as for plasma (see 3.5.4 above).

#### **3.7.5. Ultrafiltrate assay results and discussion**

The non-specific binding of naltrexone and 6 $\beta$ -naltrexol to the filtration membranes was negligible, being 0% for naltrexone at both 5 and 50 ng/ml, and 3.1% and 1.3% for 6 $\beta$ -naltrexol at 5 and 50 ng/ml, respectively. The filtered samples did have an extra peak in the chromatography at a retention time of 11 minutes, thought to be due to glycerol from the filtration devices. Therefore, the devices were washed with distilled water prior to each use, which removed the peak.

### **3.8. Simultaneous assay for naltrexone and 6 $\beta$ -naltrexol in saliva**

#### **3.8.1. Introduction**

As part of the pharmacokinetic study (see Chapter 5), saliva samples were collected from the volunteers at the same times as the blood samples. Previously, Verebey and colleagues had shown that while 6 $\beta$ -naltrexol was present in saliva in much lower concentrations than in urine, the 6 $\beta$ -naltrexol concentrations in saliva were at least ten times those of naltrexone at 16 and 24 hours after 2x200 mg oral doses of naltrexone in four subjects (Verebey et al., 1980). The variation in saliva concentrations was large, ranging from 43.5 to 571.6 ng/ml 24 hours after the dose in the four subjects tested. The mean ( $\pm$  SD) saliva concentrations (13.9 $\pm$ 17.1 and 166.7 $\pm$  184) were similar to the mean plasma concentrations (9.4 $\pm$  5.2 and 200.6 $\pm$  39.1) for naltrexone and 6 $\beta$ -naltrexol, respectively.

#### **3.8.2. Saliva assay conditions**

The assay conditions used for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol



in saliva were the same as described for plasma previously (1.5.2). The mobile phase used for the optimal separation of naloxone (internal standard), naltrexone and 6 $\beta$ -naltrexol comprised 16% methanol in 50 mM KH<sub>2</sub>PO<sub>4</sub> adjusted to a pH of 3.2 with orthophosphoric acid. The mobile phase was filtered through 0.2  $\mu$ m filters under vacuum and degassed by sonication prior to use, and the flow rate was 0.8 ml/min.

### **3.8.3. Saliva sample preparation**

Drug free saliva samples (1.0 ml) containing known concentrations of naltrexone and 6 $\beta$ -naltrexol, plus internal standard (100  $\mu$ l of 100 ng/ml naloxone) were centrifuged for 10 min at 3500, then transferred to new 10 ml flat-bottomed plastic tubes, alkalised with 500  $\mu$ l 1M Na<sub>2</sub>CO<sub>3</sub> (pH 9.0) and extracted with 5 ml dichloromethane for 30 min on a rotary mixer. After centrifuging the samples for 10 min at 3500 rpm, the upper aqueous layer was removed, and the samples were washed with an additional 500  $\mu$ l Na<sub>2</sub>CO<sub>3</sub> by vortexing for 30 sec. After centrifugation (10 min, 3500 rpm), the aqueous layer was again removed, and the organic layer was transferred to a new 10 ml tube containing 200  $\mu$ l of 50 mM KH<sub>2</sub>PO<sub>4</sub> (pH 2.0). The samples were then mixed for 30 min on a rotary mixer, centrifuged for 10 min at 3500 rpm, and 170  $\mu$ l of the acid bubble was transferred to injection vials.

### **3.8.4. Saliva assay validation**

Retention times of the compounds of interest were confirmed by direct injection of aqueous solutions of pure compounds. Quantification of naltrexone and 6 $\beta$ -naltrexol from unknown and quality control samples was performed with calibration curves consisting of eight standards over the concentration range 0.5-25 ng/ml (naltrexone) and 2-100 ng/ml (6 $\beta$ -naltrexol) as the free base. All saliva calibration and QC samples were prepared as 10-fold concentrated stocks and diluted on the day of assay in drug-free saliva. Subject samples which contained naltrexone or 6 $\beta$ -naltrexol at concentrations above the analytical limit of quantification were diluted in drug-free saliva and re-extracted.

Intra- and interday, and limit of quantification validation studies for the saliva method were not performed as the standard and quality control samples were the same as for plasma, and the assay method was the same as for plasma and urine. The ongoing performance of the assay was monitored with quality control samples and these data are summarised in Section 5.4. Naltrexone and 6 $\beta$ -naltrexol precision and accuracy were monitored with quality control (QC) samples prepared in duplicate at three concentrations: low (LQC 1.75 and 7.5 ng/ml for

the two compounds, respectively), medium (MQC 7.5 and 37.5 ng/ml) and high (HQC 17.5 and 75 ng/ml). The lower limit of quantification (LOQ) was determined by analysis of the lowest calibration standard (n=6) for each analyte in a separate assay. LOQ was determined as the lowest calibration standard that produced a precision of < 20% and an inaccuracy of < ± 20%.

Assay acceptance criteria were defined as: calibration curve  $r^2$  values greater than 0.99; 6/8 standard values and 4/6 QC values less than 20% deviation from their nominal concentration.

The extraction recoveries of the compounds of interest (naltrexone, naloxone and 6β-naltrexol) were assessed by comparison of the peak heights obtained in an aqueous solution and post-extraction of the quality control samples.

The specificity of the assay was assessed by injecting extracted drug-free saliva samples. All drug-free saliva used in the dilution of calibration standards, QC samples and baseline samples from the volunteers in the kinetic study were analysed to ensure that there were no compounds co-eluting with the compounds of interest.

### 3.8.5. Saliva assay Results and Discussion

There were no interfering peaks in the chromatograms of drug-free saliva. Figure 3.4 shows a representative chromatogram of a human saliva sample of a volunteer following 50 mg oral naltrexol. The extraction recoveries for naloxone (internal standard), naltrexone and 6β-naltrexol were all greater than 99%. Linear regressions of the calibration curves (n=7) with weighting 1/y gave mean slope values of 0.072 (CV 17.5%), and a mean correlation coefficient ( $r^2$ ) of 0.9977 (CV 0.22%) for naltrexone and, 0.062 (CV 5.2%) and 0.9978 (CV 0.22%) for mean 6β-naltrexol slope and correlation coefficient, respectively (Table 3.10).

Table 3.10. Mean Precision and accuracy of slope values for the linear regression of the calibration curves of 6β-naltrexol and naltrexone quantification in human saliva.

	N	Regression coefficient ( $r^2$ )	Regression coefficient Precision (%)	Mean	% (CV)
Naltrexone	7	0.9977	0.22	0.0724	17.60
6β-naltrexol	7	0.9970	0.22	0.0617	5.15

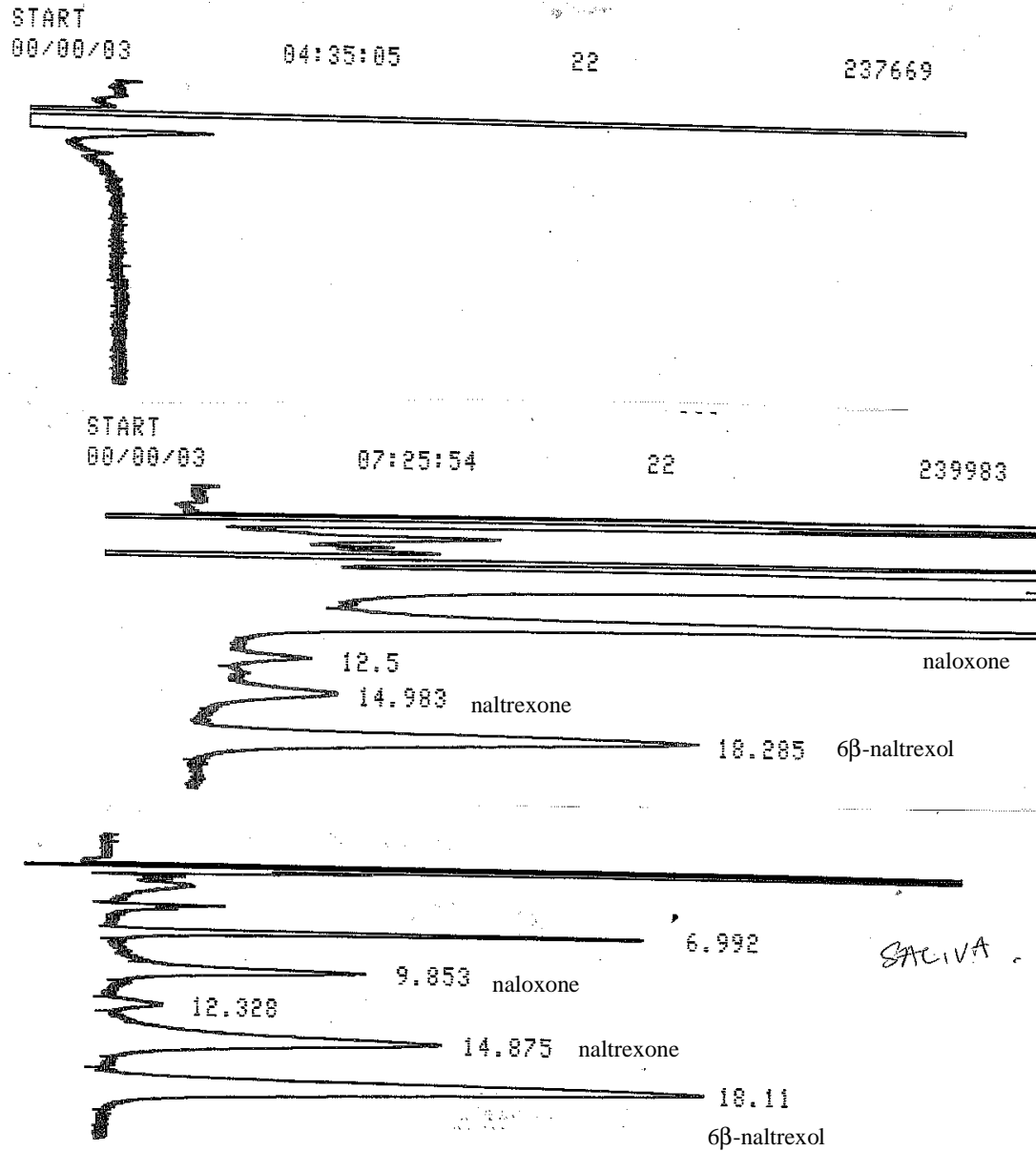


Figure 3.4 HPLC of human saliva.

Chromatogram a) blank saliva; chromatogram b) low quality control (LQC), naloxone (internal standard 10.0 min), naltrexone 1.75 ng/ml (15.0 min) and 6 $\beta$ -naltrexol 7.5 ng/ml (18.3 min); chromatogram c) volunteer sample 2.5 hr post-50 mg oral naltrexone, naltrexone 17 ng/ml (14.9 min), 6 $\beta$ -naltrexol 43.6 ng/ml (18.1 min).

### 3.9. Naltrexone and 6 $\beta$ -naltrexol analyses in whole blood

#### 3.9.1. Introduction

Whole blood samples from the pharmacokinetic study were collected prior to centrifugation in order to assess the blood to plasma concentration ratios of naltrexone and 6 $\beta$ -naltrexone after an oral dose of 50 mg or an iv dose of 1 mg of naltrexone. In an earlier study, Verebey *et al* (1980) demonstrated no distribution of 6 $\beta$ -naltrexol into red cells using a packed red cell suspension, compared to a large distribution of the non-polar metabolite HMN of 96%. This value was calculated from the difference between a benzene extraction at pH 7.5 into which 6 $\beta$ -naltrexol did not extract, and a chloroform extraction at pH 9.4 whereby both 6 $\beta$ -naltrexol and HMN were extracted. This problem arose due to their inability to separate the pentafluoropropionic anhydride (PFPA) derivatised 6 $\beta$ -naltrexol and HMN using their chromatographic system. In the same study, the blood:plasma concentration ratio of naltrexone was found to be 0.72:1. These are relative values only as the collection times were 16 and 24 hours after the previous dose of naltrexone and not continuous 24-hour collections.

#### 3.9.2. Whole blood assay conditions

The assay conditions for the analysis of whole blood samples were exactly as described for plasma, above (Section 1.5.2). The mobile phase used for the separation of naloxone (internal standard), naltrexone and 6 $\beta$ -naltrexol was also as described for the other body fluids above.

#### 3.9.3. Whole blood sample preparation

Whole blood samples were collected during the pharmacokinetic study to measure the blood:plasma concentration ratios of naltrexone and 6 $\beta$ -naltrexol. Two samples were collected (0.5 and 6 hr post-naltrexone) from each volunteer after both oral and intravenous naltrexone treatment (see 5.3.3.1). The 30 min post-naltrexone samples only were analysed. Due to the large amounts of protein in the whole blood samples, and the effect of protein on 6 $\beta$ -naltrexol recovery from plasma samples (see 3.5.6), various methods of lysis and precipitation were tested.

A drug-free whole blood sample (20 ml) was spiked with 100  $\mu$ l 1000 ng/ml naloxone and naltrexone and 100  $\mu$ l 2500 ng/ml 6 $\beta$ -naltrexol and incubated in a shaking water bath at 37°C for 60 min. Ten ml of the whole blood was centrifuged at 3500 for 10 min and the plasma and cells separated. Aliquots of plasma (1.0 ml), red cells (reconstituted with 1.0 ml physiological saline) or whole blood (1.0 ml) were treated with water (1.0 or 2.0 ml) to lyse

the cells or physiological saline (1.0 ml) and the protein was then precipitated with 1.0 or 2.0 ml acetonitrile. These samples were then extracted as outlined above for plasma (See 3.5.3.) and compared to unextracted aqueous samples to measure recovery. The study samples (1.0 ml) were treated with 1.0 ml water, and proteins were precipitated with 2.0 ml acetonitrile, and kept at 4°C overnight. The samples were then extracted as above (see 3.5.3 plasma sample preparation).

#### **3.9.4. Whole blood assay validation**

After lysing of the red cells and protein precipitation of the samples, the whole blood samples were essentially protein-free plasma, so it was not deemed necessary to revalidate these samples. The plasma validation results shown in 3.5.4 were used.

#### **3.9.5. Whole blood results and discussion**

There were some difficulties in obtaining >80% extraction efficiencies of the whole blood samples initially. These were overcome by lysing the cells first with distilled water and using twice the usual amount (1.0 ml) of acetonitrile to precipitate the proteins. The results of this work are discussed in Section 5.6.

#### **3.10. Discussion of naltrexone and 6 $\beta$ -naltrexol quantification in biological fluids**

The method described above for the quantification of 6 $\beta$ -naltrexol in cytosolic preparations using HPLC with UV detection was easily performed and had a simple extraction procedure. Shown also are relatively simple methods for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in a number of body fluids, that can be performed with good sensitivity and specificity using a simple extraction procedure and HPLC with electrochemical detection.

The assay for the quantification of 6 $\beta$ -naltrexol in cytosol using UV detection had an absolute accuracy of >87% at the limit of quantification (0.25  $\mu$ M) and all quality control concentrations (1.75, 7.5 and 14  $\mu$ M), with precision >84% for all quality control concentrations. This enabled the formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosol preparations to be quantified directly, rather than by qualitatively using radiolabelled substrate (Roerig et al., 1976) or indirectly using the oxidation of NADPH (Ohara et al., 1995).

The assay for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in human plasma also had a high degree of accuracy and precision. Extraction and assay of 1.0 ml of plasma

resulted in a limit of quantification of 0.5 ng/ml for both analytes, with intra- and inter-precisions ranging from 0.8 to 6.1% for naltrexone and 0.7 to 7.2% for 6β-naltrexol at three different concentrations. This compares favourably with a previously published method (Davidson et al., 1996) in which 2.0 ml of plasma were assayed with resulting limits of quantification of 0.25 and 0.5 ng/ml for naltrexone and 6β-naltrexol, respectively, with intra- and interday precisions ranging from 0.5 to 7.7% for naltrexone and 0.7 to 8.2% for 6β-naltrexol. One difference between the two assay procedures was the binding of 6β-naltrexol to a constituent of the plasma in the current study, which necessitated the precipitation of plasma proteins with acetonitrile prior to extraction in order to obtain a recovery of 6β-naltrexol between 73 and 92% slightly lower than those obtained for naltrexone and the internal standard naloxone, which ranged from 78-100%. Davidson *et al.*, (1996) obtained good recovery of 6β-naltrexol without the need to precipitate the samples initially. This may be due to differences in the sample collection tubes or assay tubes used in the two studies.

The assay for the simultaneous quantification of naltrexone and 6β-naltrexol in human urine extracting 100 µl of sample also performed with a high degree of accuracy and precision. The only problems encountered related to the high concentrations of 6β-naltrexol in both hydrolysed and unhydrolysed urine samples, which necessitated an adaptation of the method used for the other fluids.

The assays for the simultaneous quantification of naltrexone and 6β-naltrexol in saliva, whole blood and ultrafiltrate were not fully validated in the current study (intra- and interday and limit of quantification, and intraday precision and accuracy studies not performed). This decision was based on the assumption, rightly or wrongly, that the saliva and ultrafiltrate samples would have similar matrices to the aqueous assay, whereas the whole blood samples following cell lysis and protein precipitation with acetonitrile would resemble the plasma samples.

While the assay used in the current study for the quantification of naltrexone may not be as sensitive as some of those described earlier (Davidson et al., 1996; Meyer et al., 1984; Reuning et al., 1989; Wall et al., 1984), radiolabelled compounds were avoided in the current study, and the samples did not have to undergo complicated extraction and derivatisation procedures. The assay was sensitive enough (with 1.0 ml sample volume) to measure analytes in various biological fluids obtained in the kinetic study reported below.

## 4. Kinetics and Inhibition of the Formation of 6 $\beta$ -Naltrexol from Naltrexone in Human Liver Cytosol

### 4.1. Introduction

Although 6 $\beta$ -naltrexol has been known for many years to be quantitatively the major metabolite of naltrexone in humans (Dayton and Inturrisi, 1976), few studies have examined the enzyme(s) involved, and the *in vitro* kinetics of its formation. Malspeis *et al* (1976) examined the metabolic reduction of naltrexone from guinea pig, rats and monkey livers. Using 9000 g and 15000 g supernatants and microsomal fractions of liver from these species, incubated with an NADPH-generating system, they reported that naltrexone was reduced to both  $\alpha$ - and  $\beta$ -naltrexol in the guinea pig liver preparations. In contrast to this, the monkey liver study yielded predominantly 6 $\beta$ -naltrexol with a 5-16% concentration-independent formation of  $\alpha$ -naltrexol. The rat liver preparations reduced naltrexone much less efficiently with a trace amount of 6 $\beta$ -naltrexol produced and no 6 $\alpha$ -naltrexol. These formation products were analysed qualitatively by gas-liquid chromatography. Additionally, when  $\alpha$ - and  $\beta$ -naltrexol were incubated in the 9000 g supernatants of guinea pig liver with the NADPH-generating system, no epimerisation of metabolites was observed (Malspeis *et al.*, 1976).

In a further animal study, Roerig and colleagues (1976) characterised the enzymes responsible for the reduction of naloxone and naltrexone in rabbit and chicken liver. These species were chosen as it was known that the chicken reduces these ketones to the 6 $\alpha$ -hydroxy product, whereas the rabbit reduction products are in the form of the 6 $\beta$ -hydroxy epimers. Ammonium sulphate precipitated fractions of 100000g supernatant fractions of liver preparations were incubated with a NADPH-generating system in the presence of radiolabelled ( $^{14}\text{C}$  for naloxone,  $^3\text{H}$  for naltrexone) and unlabelled substrate. The incubates were then extracted with ethyl acetate, 400  $\mu\text{l}$  of which was spotted onto silica-gel TLC plates. The  $K_m$  values for the reduction of naltrexone in the rabbit and chicken livers were  $5.4 \times 10^{-4}$  M and  $3.0 \times 10^{-4}$  M, respectively. The  $V_{\text{max}}$  values are not reported.

These researchers also conducted inhibition studies using morphine, methadone, ketamine and dihydromorphinone. The rabbit liver enzymes were inhibited by morphine and methadone at concentrations of  $0.1\text{-}2.0 \times 10^{-3}$  M whereas the chicken liver enzymes were only inhibited by ketamine over this concentration range. Dihydromorphinone inhibited both enzyme systems, with the rabbit liver being more sensitive (Roerig *et al.*, 1976).

Ohara and co-workers, using purified enzyme preparations from autopsied human liver

samples, showed stereospecific reduction of naltrexone to 6 $\beta$ -naltrexol by dihydrodiol dehydrogenases, now termed aldo-keto reductases (AKR1 family), (DD1 [AKR1C1] pI 9.1; DD2 [AKR1C2] and DD4 [AKR1C4], pI 5.4), but not carbonyl reductase (EC 1.1.1.184), nor aldehyde reductase (EC 1.1.1.21) (Ohara et al., 1995). Using cloning techniques, DD4 was found to be identical to human 3 $\alpha$ -hydroxysteroid dehydrogenase, which was identical to chlordecone reductase (Binstock et al., 1992), but different from carbonyl reductase (Wermuth et al., 1988). The family of dihydrodiol dehydrogenases (DDs 1-4) showed a broad range of substrate specificity in ketone reduction activity, indicating structurally distinct carbonyl-reducing enzymes in the liver (Ohara et al., 1995). However, in this study, quantification of 6 $\beta$ -naltrexol was not performed, as formation was indirectly assessed by measuring the oxidation rate of NADPH at 340 nm, whilst reaction products were identified using thin layer chromatography by comparison with pure 6 $\alpha$ - and 6 $\beta$ -naltrexol.

There are few therapeutic drugs that undergo a similar metabolic pattern to naltrexone. The antipsychotic drug haloperidol has also been shown to be reduced by cytosolic carbonyl reductase and to a lesser extent by DD1 and DD2 (Ohara et al., 1995); the reverse reaction (reduced haloperidol to haloperidol) has been shown to be carried out by microsomal cytochrome P450s (Korpi et al., 1985), specifically CYP2D6 (Tyndale et al., 1991), and CYP3A4 (Kudo and Odomi, 1998; Pan, 1998). To date it has not been shown whether the reverse reaction of 6 $\beta$ -naltrexol to naltrexone can also occur in humans.

#### **4.2. Aims**

The aims of the current study were to:

- 1) Determine the enzyme kinetics for the formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosolic preparations;
- 2) Characterise the possible enzyme(s) involved using chemical inhibitors, including likely concomitantly taken drugs, general reductase inhibitors, and some steroid compounds;
- 3) Determine the specificity of cytosolic compared to microsomal enzymes in the formation of 6 $\beta$ -naltrexol from naltrexone, and whether the reverse reaction (6 $\beta$ -naltrexol to naltrexone) occurs in microsomal preparations.

The assay developed for the quantification and validation of naltrexone and 6 $\beta$ -naltrexol in human liver cytosol as described in the previous chapter was used to help achieve these aims.



### 4.3. Methods

#### 4.3.1. Introduction

This study was approved by the Committee on the Ethics of Human Experimentation of the University of Adelaide and the Human Ethics Committee of the Royal Adelaide Hospital. Patients gave written informed consent for their liver tissue to be used following cholecystectomy or partial hepatectomy. The liver tissue samples used [HLS# 11, 15, 18, 19, 21, 22, 24 and 31 (internal numbers)] were from donors ranging in age from 41 to 73 years, of whom four were female, and four male. The patient demographics and laboratory results are given below (Table 4.1).

#### 4.3.2. Chemicals

The chemicals used in this study and their sources of supply are described in Section 3.3 of Chapter 3. The 6 $\beta$ -naltrexol was synthesised as the free base in the Department of Chemistry, University of Adelaide by the method of Chatterjie *et al.*, (1975). The purity of the 6 $\beta$ -naltrexol used for standard and quality control preparation was as described in Chapter 2.

Additional compounds used in the inhibition of 6 $\beta$ -naltrexol formation in cytosol studies were haloperidol, chlorpromazine, finasteride and nicotine all from Sigma Aldrich (St Louis, Mo, USA), testosterone, dihydrotestosterone and corticosterone (gifts from Professor Howard Morris, Department of Clinical Biochemistry, Institute of Medical and Veterinary Science, Adelaide, Australia). Temazepam, oxazepam and lorazepam were obtained from Wyeth Pharmaceuticals GmbH (Munster, Germany), flunitrazepam from Roche Products (Dee Why, Australia), diazepam was a gift from Professor John Miners, Flinders Medical Centre (Adelaide, Australia), and fluoxetine from Associate Professor Wayne Hooper, Department of Medicine, Royal Brisbane Hospital (Australia). Phenobarbitone sodium was purchased from Faulding Australia (Adelaide, Australia).

#### 4.3.3. Preparation of cytosol and microsomes

Cytosol was prepared as a by-product of the preparation of microsomes by differential centrifugation of liver homogenate (Zanger *et al.*, 1988). Small sections of liver tissue of known weight were homogenised in 2.4 ml/g of microsome preparation buffer (1 mM EDTA in 0.15 M KCl, pH 7.3), using a mechanical homogeniser (Thyristor Regler, John Morris Scientific Instruments Pty Ltd, Sydney, Australia). The homogenate was then filtered through gauze pads, and rehomogenised with a teflon plunger (0.2 mm clearance) in a glass potter. Aliquots of the homogenate were then centrifuged (12000 *g*, 15 min) at 4 °C (J2-21

centrifuge, JA 20 rotor, Beckman, CA, USA). The pellets were discarded and the supernatant recentrifuged (27000 g, 15 min) at 4 °C. The pellets were again discarded and the supernatant was recentrifuged (105000 g, 60 min) at 4 °C (L7-55 Ultracentrifuge, 70.1, Ti-rotor, Beckman). The resulting supernatant contained the cytosolic fraction, and was retained. The pellets were again homogenised in 2-3 ml of washing buffer (1mM EDTA, 0.1 M sodium pyrophosphate decahydrate, pH 7.25) in a glass-glass potter. Aliquots were then centrifuged (105000 g, 60 min) at 4°C (L7-55 Ultracentrifuge, 70.1, Ti-rotor, Beckman). The supernatant cytosolic fraction was collected and pooled with the previously collected sample and assayed for protein content. Aliquots of the cytosolic fraction (200 µl) were placed in Eppendorf tubes. The pellets containing the microsomal fraction were rehomogenised in a glass-glass potter with a volume of microsomal storage solution (1mM EDTA, 0.1 M sodium orthophosphate, pH 7.4) corresponding to 1 ml/g of original liver sample weight. Aliquots (200 µl) were placed in Eppendorf tubes. Liver samples, cytosol and microsomes were stored at -80 °C until used.

#### **4.3.4. Measurement of cytosolic and microsomal protein concentrations**

The cytosolic and microsomal samples were analysed for protein concentration (Lowry et al., 1951). The samples were diluted (1/25 and 1/50) with microsomal storage solution and assayed together with six calibration standards (0, 50, 100, 200, 400 and 800 µg/ml) of bovine serum albumin (fraction V) prepared in duplicate. Samples were then mixed with 2 ml solution 1 (0.02% CuSO<sub>4</sub>, 0.4% K<sup>+</sup>-Na<sup>+</sup>-tartrate, 2.9% Na<sub>2</sub>CO<sub>3</sub> and 0.39% NaOH) by briefly vortexing. They were left to stand for 10 min, then 200 µl of Solution 2 (1:2 dilution of Folin-Ciocalteu) reagent in water was added. After briefly mixing, the samples were left to stand in darkness for 20-30 min. The absorbance at 550 nm was measured using a double-beam spectrophotometer (U-2000, Hitachi, Tokyo, Japan). Calibration standards were used to calibrate the spectrophotometer, and the total protein content of the cytosolic and microsomal samples was calculated. The cytosolic samples were diluted in phosphate buffer (100 mM, pH 7.4) to 0.5 mg of protein/ ml for use as stock for the assays.

#### **4.3.5. HPLC Conditions**

The formation of 6β-naltrexol from naltrexone in human liver cytosol was quantified using a reversed phase HPLC system as described in 3.4.2.

#### **4.3.6. 6β-naltrexol quantification**

Retention times for naltrexone and 6β-naltrexol were 13 and 16 min, respectively. The

ongoing performance of the assay was monitored with quality control samples assayed in duplicate at concentrations of LQC 1.75  $\mu$ M, MQC 7.5  $\mu$ M and HQC 14  $\mu$ M. In order for an individual assay to be accepted it had to fulfil the acceptance criteria as described in Chapter 3, section 3.4.6 ( $r^2 > 0.99$ , 4/6 QC's and 6/8 standards within 20% of the nominal concentration).

#### 4.3.7. Time-dependency studies

Prior to analyses of the rate of formation of 6 $\beta$ -naltrexol from naltrexone in these cytosolic studies, the optimum incubation time of the cytosolic preparations had to be determined. The linearity of the formation of 6 $\beta$ -naltrexol over time was assessed by incubating HLS #31 in duplicate for various time points up to two hours (0, 20, 40, 60, 80, 100 and 120 min) with 60  $\mu$ M naltrexone (50  $\mu$ l of 240  $\mu$ M) and 25  $\mu$ g of cytosolic protein (50  $\mu$ l of 0.5 mg/ml). All other assay conditions were as described below in section 4.3.9.

#### 4.3.8. Protein-dependency studies

To further optimise the conditions for the formation of 6 $\beta$ -naltrexol from naltrexone in these cytosolic studies, the optimum concentration of cytosolic protein had to be determined. This was performed by incubating substrate (naltrexone, 40  $\mu$ l of 60  $\mu$ M) with 50  $\mu$ l of cytosol (0.025 to 7.0 mg/ml of protein). An internal standard (naloxone, 10  $\mu$ l of 100  $\mu$ M) was used to compare the peak height ratios to 6 $\beta$ -naltrexol. These studies were initially conducted using an NADPH-generating system (50  $\mu$ l comprised of 2 mM NADP, 20 mM glucose-6-phosphate, 20 mM MgCl<sub>2</sub> in 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) and 50  $\mu$ l glucose-6-phosphate dehydrogenase (40 U/ml), which as shown below (section 4.4.3) resulted in the formation of 6 $\alpha$ -naltrexol. The studies were repeated using NADPH as the enzyme co-factor (100  $\mu$ l of 140  $\mu$ M), 50  $\mu$ l of cytosol (protein 0.0625-10 mg/ml), 50  $\mu$ l naltrexone (60  $\mu$ M) with all other conditions as described in 4.3.9. No internal standard was used in the assay, and protein concentration was correlated with 6 $\beta$ -naltrexol peak heights. The liver tissue used for all studies was HLS#31.

#### 4.3.9. Cytosolic incubations

Examination of the optimal conditions for the cytosolic incubations resulted in samples being performed in duplicate at 37°C in a shaking water bath (Julabo SW20-C, John Morris Scientific Pty, Ltd Sydney, Australia) for 60 min. In initial studies, the substrate concentration range was 2-250  $\mu$ M, but the linearity of the Eadie-Hofstee plots was reduced at

the higher substrate concentrations, and in a previous study in this laboratory (unpublished results), the maximum substrate concentration used was 5-150  $\mu\text{M}$ , so the assays were performed with a maximum naltrexone concentration in the assay of 150  $\mu\text{M}$ . The incubates of 200  $\mu\text{l}$  final volume contained 100 mM phosphate buffer (pH 7.4), 140  $\mu\text{M}$  NADPH, substrate (naltrexone) concentrations of 5, 10, 15, 25, 60, 60, 75, 100 and 150  $\mu\text{M}$ , and 25  $\mu\text{g}$  of cytosolic protein. The addition of 100  $\mu\text{M}$  of inhibitor to the incubation mix was the only difference for the inhibitor studies. The enzymatic formation of 6 $\beta$ -naltrexol was terminated by the addition of 100  $\mu\text{l}$  of ice-cold methanol. The samples were then vortexed briefly, centrifuged for 10 min at 14000 rpm, and 100  $\mu\text{l}$  of the supernatant was injected onto the HPLC system.

Calibration curves and quality control samples were run with every assay to determine the formation of 6 $\beta$ -naltrexol from naltrexone and to monitor the validity of the assay. The assay validation data are reported in 3.4.5.

#### **4.3.10. Microsomal incubations**

Incubations were performed as above, and the incubates of 200  $\mu\text{l}$  final volume contained 100 mM phosphate buffer (pH 7.4), NADPH-generating system (1 mM NADP, 1 U/ml isocitrate dehydrogenase, 5 mM isocitric acid, and 5 mM  $\text{MgCl}_2$ ), substrate (naltrexone 15, 60 and 150  $\mu\text{M}$  or 6 $\beta$ -naltrexol 20, 60, 150, 250, 500 and 1000  $\mu\text{M}$ ), and 25  $\mu\text{g}$  of microsomal protein from one of the above livers (HLS#31). The incubation conditions using this NADPH-generating system have been used extensively in this laboratory previously (Foster et al., 1999).

#### **4.3.11. Inhibition studies with chemical inhibitors**

Cytosolic preparations from three human liver samples (HLS# 11, 15 and 31) were used in triplicate to examine the inhibition of 6 $\beta$ -naltrexol formation. The naltrexone concentration for the initial inhibitor studies was 30  $\mu\text{M}$ , the approximate  $K_m$  value for the liver samples used. Inhibitors were incubated initially at 100  $\mu\text{M}$ , and for those compounds that showed greater than 50% inhibition,  $K_i$  values were determined. Prior to this, these inhibitors were incubated at various concentrations with 30  $\mu\text{M}$  of substrate to assess concentration-dependency. The  $K_i$  determinations were conducted by incubating the inhibitors at four different concentrations (5, 10, 25 and 50 mM for naloxone, menadione and corticosterone; 0.1, 0.5, 1.0 and 5 mM for testosterone and dihydrotestosterone) with three different substrate

concentrations (25, 50 and 100  $\mu$ M). The latter two studies were again performed in duplicate using the samples HLS#'s 11, 15 and 31.

Various inhibitors were tested on the basis that they were general or potential reductase inhibitors, or they could be co-administered to people for whom naltrexone was prescribed. Nicotine, morphine, methadone, oxycodone, naloxone, hydromorphone and oxymorphone (pH 7.4), and warfarin (pH 9.0) were dissolved in phosphate buffer. The benzodiazepines lorazepam, temazepam, oxazepam, diazepam and flunitrazepam as well as chlorpromazine, phenobarbitone, fluoxetine, 3-trifluoromethylphebylpropan-2-one (a metabolite of the antiarrhythmic flecainide), 2-chlorosyringaldehyde and menadione were dissolved in 5% methanol (final concentration 0.625% in assay). Haloperidol was dissolved in 25% methanol (final concentration in assay 3.125%). The steroid hormones testosterone, dihydrotestosterone and corticosterone, and finasteride (a  $5\alpha$ -reductase inhibitor) were dissolved in 10 % ethanol (1.25% in assay).  $\beta$ -Oestradiol, progesterone, prednisone, prednisolone, hydrocortisone and cortisone were dissolved in 45% ethanol. Stilboestrol was dissolved in 45% methanol. Incubations containing equivalent amounts of diluent were always used as controls and, in addition, control incubations containing inhibitors alone were used to confirm that the inhibitors did not produce any chromatographic peaks that could interfere with the quantification of  $6\beta$ -naltrexol. Assay conditions for the inhibitor studies did not differ from those of the kinetic study.

#### **4.3.12. Problems encountered with cytosolic incubations and inhibitor studies**

Previous studies investigating the reduction of carbonyl compounds in liver from various species have used either an NADPH-generating system (comprising  $\text{NAD(P)}^+$ , a  $\text{H}^+$  donor system and a magnesium-containing buffer), (Moreland and Hewick, 1975; Pollock, 1975; Roerig et al., 1976), or NAD(P)H itself (Ohara et al., 1995; Wong et al., 1992). However, using the NADPH-generating system which comprised 2 mM NADP, 20 mM glucose-6-phosphate, 20 mM  $\text{MgCl}_2$  in 0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 7.4) and 50  $\mu$ l glucose-6-phosphate dehydrogenase (40 U/ml), a protein-independent formation of  $6\alpha$ -naltrexol was evident. Hence, NADPH only was used in the current study.

Solubility problems were encountered for some of the compounds used in the inhibitor studies. When dissolved in 45% ethanol or 45% methanol, the peak heights for the controls were very low, and the testing of the compounds involved was abandoned due to time constraints. Those compounds affected were progesterone, prednisone, prednisolone, Susan J Porter, *PhD Thesis, 2010*

hydrocortisone, cortisone and  $\beta$ -oestradiol. The steroid stilboestrol, and the ketone 3-trifluoromethylphenylpropan-2-one were oily liquids which formed oily suspensions and which resulted in chromatograms containing numerous interfering peaks.

#### 4.3.13. Data analyses

The rates of formation ( $V$ ) of  $6\beta$ -naltrexol from the substrate naltrexone concentration ( $C$ ) were expressed as  $\text{nmol mg}^{-1} \text{ protein h}^{-1}$ , and Eadie-Hofstee ( $V/\text{Substrate concentration vs } V$ ) plots were constructed. One-enzyme (1), and two-enzyme (2), Michaelis-Menten equations, with weighting  $1/y$ , were fitted to the data using nonlinear least-squares regression analysis (Regression, Blackwell Scientific Publications, Oxford UK) giving estimates of  $V_{\max}$  and  $K_m$ , where  $V_{\max}$  is the maximum reaction velocity and  $K_m$  is the Michaelis-Menten constant, which is the substrate concentration at which the reaction rate is half  $V_{\max}$ . Intrinsic clearance ( $Cl_{\text{int}}$ ) was calculated as  $V_{\max}/K_m$ :

$$V = \frac{V_{\max} * C}{K_m + C} \quad (1)$$

$$V = \left( \frac{V_{\max 1} * C}{K_{M1} + C} \right) + \left( \frac{V_{\max 2} * C}{K_{M2} + C} \right) \quad (2)$$

Inhibition was expressed as mean  $\pm$  SD, and  $K_i$  values for those inhibitors tested were determined by fitting different types of inhibitor models (competitive (3), non-competitive (4), uncompetitive (5)) to the data:

$$V = \frac{V_{\max} * C}{(K_m * (1 + I / K_i)) + C} \quad (3)$$

$$V = \frac{V_{\max} * C}{(K_m * (1 + I / K_i)) + (C * (1 + I / K_i))} \quad (4)$$

$$V = \frac{V_{\max} * C}{K_m + (C * (1 + I / K_i))} \quad (5)$$

where  $I$  = inhibitor concentration, and  $K_i$  = inhibition constant. The choice of model was

based on visual inspection of the goodness of fit of the observed data to those predicted, a significant reduction in the weighted sum of squared deviations, and random distribution of the scatter of observed data points about the fitted curve (Mikus et al., 1991). Paired t-tests comparing 6 $\beta$ -naltrexol formation in the presence of each inhibitor to the uninhibited formation were performed to determine if the differences in formation were significant. All data are expressed as mean  $\pm$  SD.

#### 4.4. Results

##### 4.4.1. Validation and on-going performance of the assay

Table 4.1 shows the patient demographics and laboratory results for the donors of the eight liver samples used in this study. Pre-operative biochemistry and haematology levels were within the normal range for all patients, except patient #15 (serum alkaline phosphatase and alanine aminotransferase, four and two-fold the upper reference limit, respectively); patient #19 ( $\gamma$ -glutamyl transferase three times the upper limit); patient #31 showed abnormal haematology with a decreased haemoglobin (4 g dl<sup>-1</sup> below the male reference limit), and increased white cell and platelet counts (20% above normal). The kinetic parameters of 6 $\beta$ -naltrexol formation for these three patients were similar to the values from those patients who exhibited no biochemical or haematological abnormalities.

The validation data for the assay used in this study are reported in section 3.4.5. The results for the ongoing performance of the assay are given below in Table 4.2. The precision of the quality controls was <5% at all levels, with absolute accuracies of >98%. The regression coefficient ( $r^2$ ) for all calibration curves was greater than 99%.

Chapter 4: In vitro Naltrexone kinetics

Table 4.1: Patient demographics and laboratory results of the liver sample donors. ( $\uparrow$  indicates markedly increased concentration,  $\downarrow$  indicates markedly decreased concentration). WBC=white blood cells, Hb=haemoglobin, Alb=albumin, ALT=alanine-aminotransferase, AST=aspartate-aminotransferase, LDH=lactate dehydrogenase, GGT= $\gamma$ -glutamyltransferase and ALP=alkaline phosphatase.

HLS#	AGE	SEX	Usual Medication	Haematology Results			Biochemistry Results					
				WBC	Hb	Plt	Alb	ALT	AST	LDH	GGT	ALP
11	41	F	Digesic, temazepam	21.3 $\uparrow$	16.4	220	42	-	21	192	17	84
15	51	M	Cephalexin, paracetamol//codeine	9.3	13.3	326	41	122 $\uparrow$	43	207	51	477 $\uparrow$
18	65	F	Diazepam, thyroxine temazepam	6.1	13.6	293	44	23	14	179	38	118
19	45	M	Allopurinol	11.2	13.1	219	40	-	25	212	206 $\uparrow$	98
21	70	M	Nil	9.3	14.2	205	42	11	11	219	29	93
22	54	F	Pyridoxine, cephalothin metronidazole, entamycin, microlax2, promethazine, hydrocortisone	5.2	13.3	218	43	37	12	179	-	98
24	42	F	Temazepam	7.4	13.8	361	43	21	15	187	32	101
31	73	M	Frusemide	17.8 $\uparrow$	10.6 $\downarrow$	270	33 $\downarrow$	10	-	-	21	105
<b>Reference Intervals</b>				<b>4-11</b>	<b>13.5-18</b>	<b>150-400</b>	<b>34-38</b>	<b>0-55</b>	<b>0-45</b>	<b>110-230</b>	<b>0-60</b>	<b>30-110</b>



Table 4.2: Inter-assay accuracy and precision for the ongoing performance of the assay for the quantification of 6 $\beta$ -naltrexol in human liver cytosol. LQC, MQC and HQC refers to the low, mid and high quality controls, respectively.

	n	Nominal Conc. ( $\mu$ M)	Mean % Accuracy ( $\pm$ SD)	%Coefficient of Variation
LQC	20	1.75	99.8 (3.0)	2.86
MQC	20	7.5	99.9 (3.1)	3.18
HQC	20	14	98.5 (2.99)	3.05

#### 4.4.2. Time-dependency studies

The rate of formation of 6 $\beta$ -naltrexol from naltrexone (60  $\mu$ M), was found to be linear up to 120 minutes (Figure 4.1). The samples for both the kinetic and inhibitor studies were all incubated for 60 minutes at 37°C.

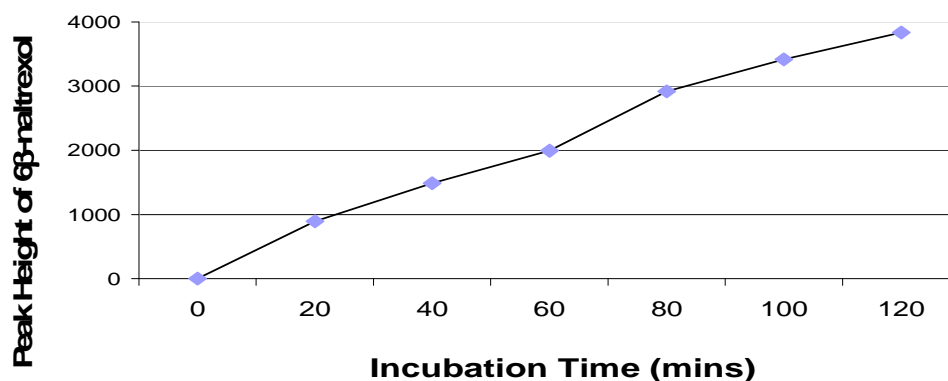


Figure 4.1 Time-dependency of the formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosol at a naltrexone concentration of 60  $\mu$ M.

#### 4.4.3. Protein-dependency studies

The initial protein dependency study conducted using the NADPH-generating system resulted in formation of 6 $\beta$ -naltrexol with a regression coefficient of 0.878 (Figure 4.2(a)). Under the conditions used for the kinetic and inhibitor studies (using NADPH), the 6 $\beta$ -naltrexol formation from naltrexone was shown to be curvilinear to 10 mg/ml of cytosolic protein with a regression coefficient of 0.999 (Figure 4.2(b)). A protein concentration of 0.5 mg/ml (25  $\mu$ g

in assay) was chosen for all studies.

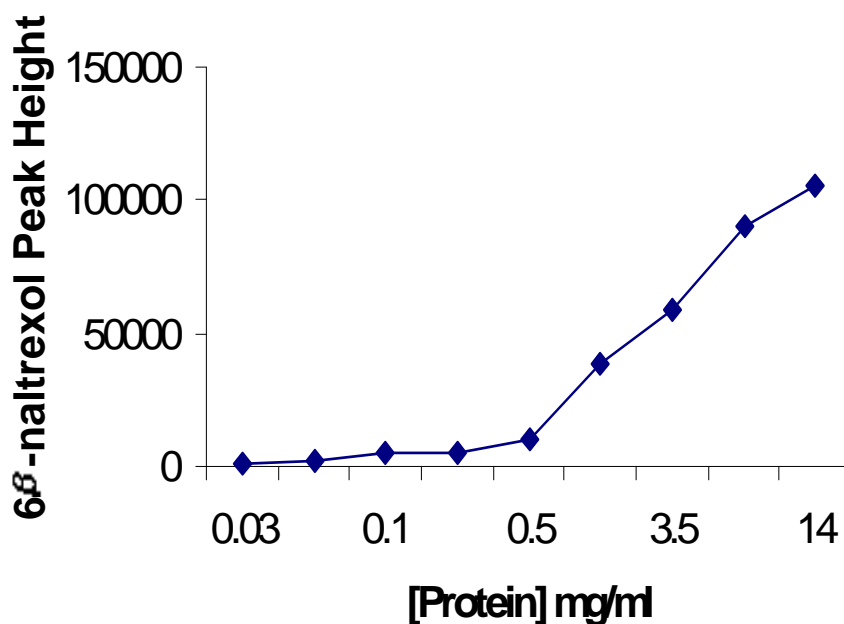


Figure 4.2(a) Initial protein-dependency study of 6β-naltrexol formation using NADPH-generating system in human liver cytosol #31

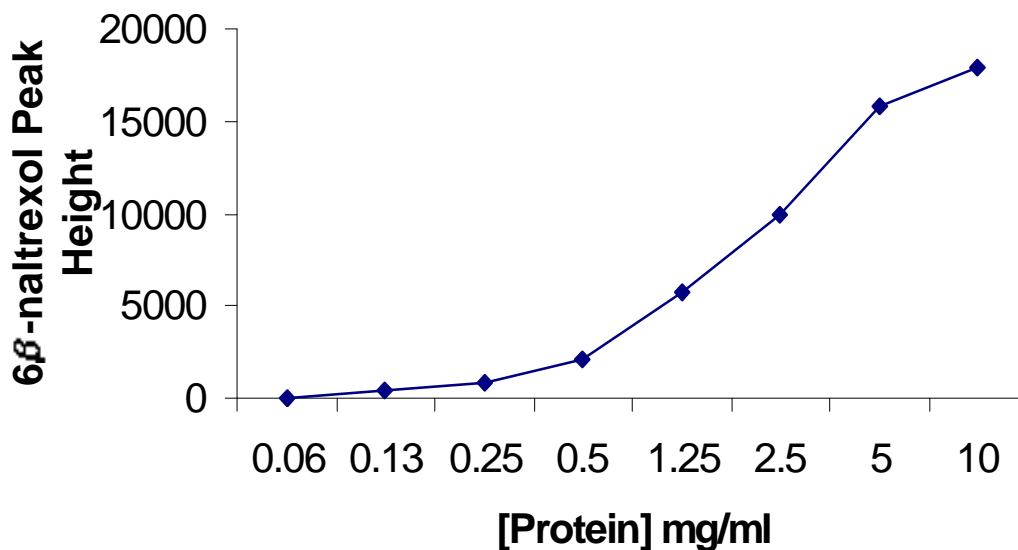


Figure 4.2(b) Protein-dependency study of 6β-naltrexol formation using NADPH (100μl of 140μM) (rather than the NADPH-regenerating system) in human liver cytosol #31

#### 4.4.4. Solutions to problems encountered in cytosol incubations

The substrate (naltrexone) concentration-dependent formation of 6 $\alpha$ -naltrexol in the cytosol incubations was not evident when NADPH was used as the co-factor in the incubations. In the inhibitor studies, it was decided not to continue further with those substances that were soluble only in 45% ethanol due to time constraints. No further studies were conducted with stilboestrol or the ketone 3-trifluoromethylphenylpropan-2-one.

#### 4.4.5. Kinetics of 6 $\beta$ -naltrexol formation from naltrexone

Eadie-Hofstee plots were found to be linear (Fig. 4.3a) and the one-enzyme Michaelis-Menten (Figure 4.3b) kinetic equation was substantially superior to the two-enzyme model. Table 4.3 shows the individual  $V_{\max}$ ,  $K_m$ , and  $Cl_{int}$  values for the formation of 6 $\beta$ -naltrexol from naltrexone for the eight human liver cytosol preparations. The variability in  $V_{\max}$  was 2.9-fold, with a range of 15.8 to 45.6 nmol/mg protein/hr. There was a larger variation in  $K_m$  of 3.2-fold, with values ranging from 17.1 to 55.45  $\mu$ M. The variation in the intrinsic clearance was 7-fold, with values of 0.29 to 2.23 ml/hr<sup>-1</sup>/mg protein.

There was no formation of 6 $\beta$ -naltrexol from naltrexone in a microsomal preparation, nor was there any naltrexone produced when 6 $\beta$ -naltrexol was incubated with microsomes and an NADPH-generating system.

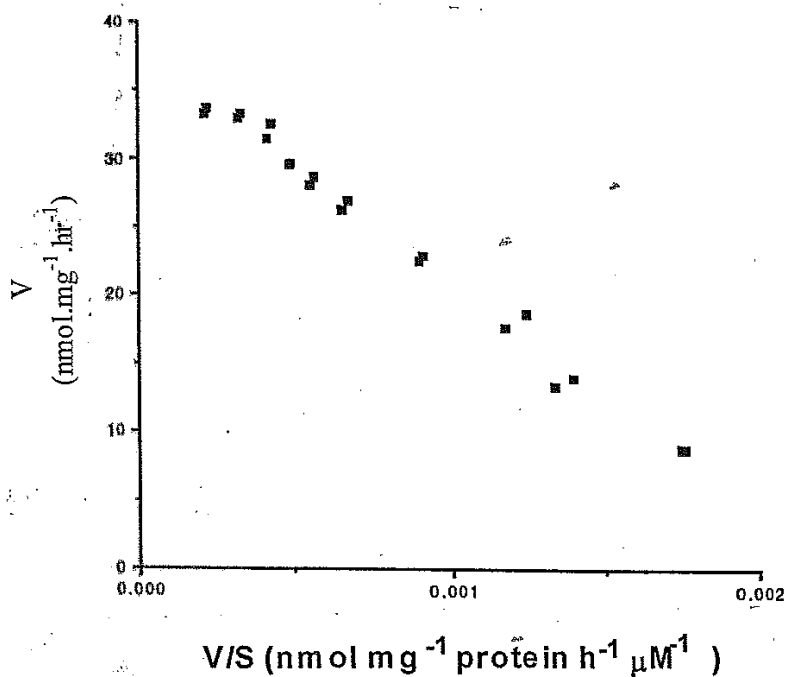


Figure 4.3a Eadie-Hofstee representation of the formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosol # 22

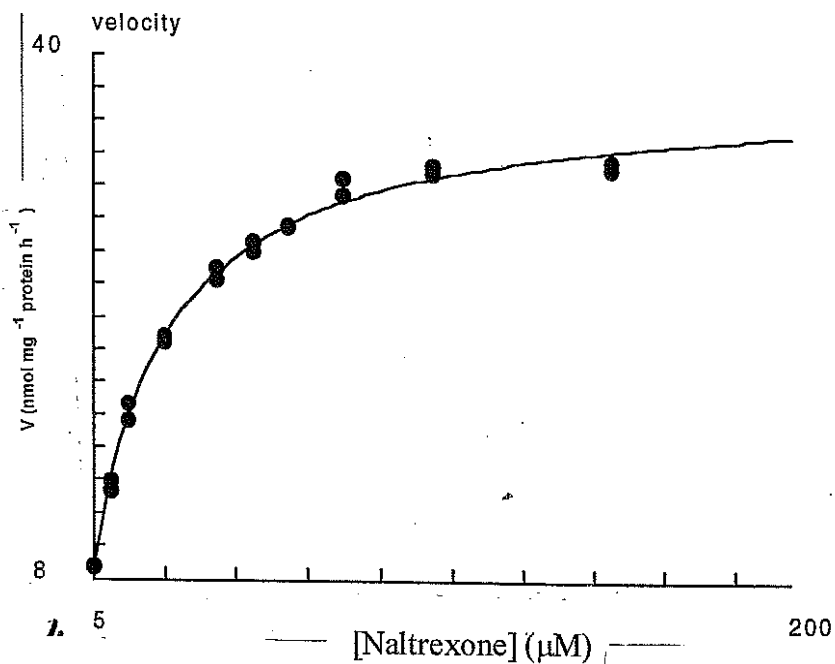


Figure 4.3b Michaelis-Menten representation of the formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosol # 22

Table 4.3 Enzyme kinetics of the formation of 6 $\beta$ -naltrexol from naltrexone using human liver cytosol preparations from 8 patients. (\* $Cl_{int} = V_{max}/K_m$ )

Human Liver Sample #	$V_{max}$ (nmolmg <sup>-1</sup> protein hr <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$Cl_{int}^*$ (mlhr <sup>-1</sup> mg <sup>-1</sup> protein)
11	30.9	21.6	1.43
15	29.3	17.8	1.65
18	16.8	52.5	0.32
19	16.7	19.2	0.87
21	15.8	55.1	0.29
22	38.2	17.1	2.23
24	18.9	48.5	0.39
31	45.6	43.3	1.05
<b>Mean</b>	<b>26.5</b>	<b>34.4</b>	<b>1.03</b>
<b>SD</b>	<b>11.3</b>	<b>16.9</b>	<b>0.70</b>
<b>% CV</b>	<b>42.6</b>	<b>49.1</b>	<b>70.0</b>

#### 4.4.6. Inhibition with chemical inhibitors

Figure 4.4 summarises the data using 100  $\mu$ M inhibitor concentrations. Fluoxetine co-eluted with 6 $\beta$ -naltrexol, therefore inhibition by fluoxetine could not be assessed and data are not included. The aldehyde, 2-chlorosyringaldehyde (2-CSA) in the incubation mixture (100  $\mu$ M) resulted in a mean inhibition of 6 $\beta$ -naltrexol of 70.8 $\pm$ 4.8% (not included in Figure 4.4) for the three cytosol preparations tested however, there was insufficient compound to conduct concentration-effect or  $K_i$  studies. Naloxone, menadione, testosterone, dihydrotestosterone and corticosterone produced greater than 50% inhibition of 6 $\beta$ -naltrexol formation ( $p < 0.001$ ). Nicotine, morphine, methadone, oxycodone, oxymorphone, hydromorphone, lorazepam, flunitrazepam, oxazepam, chlorpromazine, phenobarbitone and finasteride produced less than 20% inhibition. Warfarin, haloperidol, diazepam and temazepam inhibition values were between 20 and 40%, but only warfarin and diazepam significantly reduced 6 $\beta$ -naltrexol formation compared to control. Figure 4.5 (a-c) show representations of the concentration-dependent inhibition of 6 $\beta$ -naltrexol formation by testosterone, dihydrotestosterone and corticosterone. Naloxone and menadione also showed concentration-dependent inhibition of the formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosol preparations. The competitive inhibition model was found to be the most appropriate model for all inhibitors (using the sum of squares of the model fit) as shown in Table 4.4. The  $K_i$  values for the most potent inhibitors (naloxone, corticosterone, menadione, testosterone and dihydrotestosterone) are shown in Table 4.5.

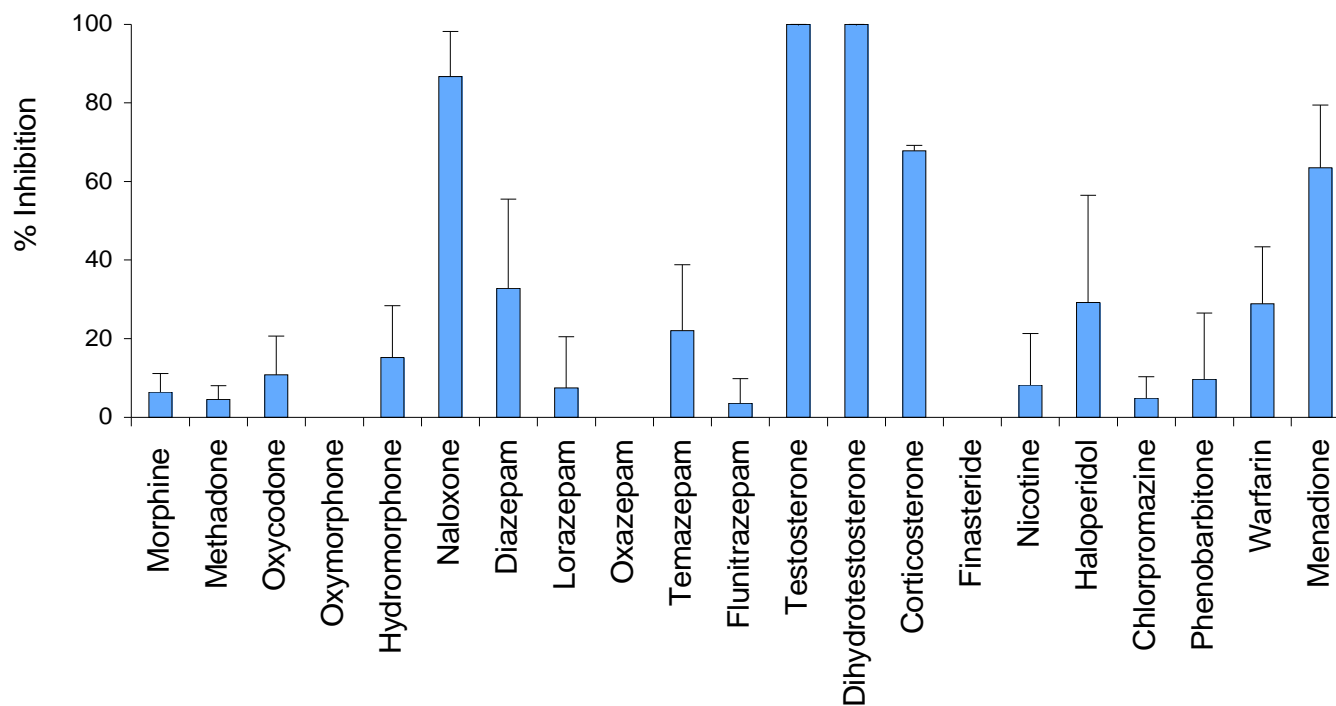
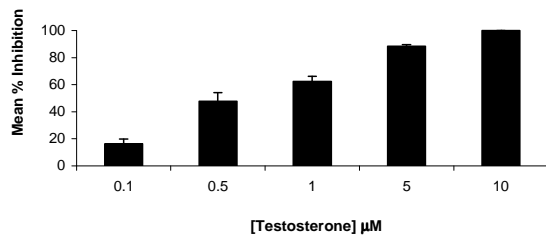
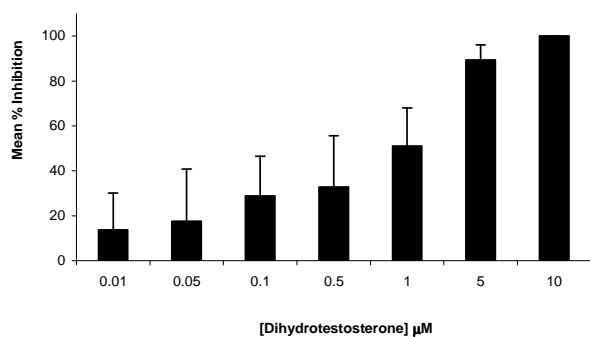


Figure 4.4. The effect of chemical inhibitors ( $100\mu\text{M}$ ) as grouped into opioids (morphine to naloxone), benzodiazepines (diazepam to flunitrazepam), steroids (testosterone to corticosterone) and miscellaneous (finasteride to menadione), on the formation of  $6\beta$ -naltrexol from naltrexone ( $30\mu\text{M}$ ) by human liver cytosolic enzymes prepared from 3 different livers. Values represent mean  $\pm$  SD. \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to control.

a) Testosterone



b) Dihydrotestosterone



c) Corticosterone

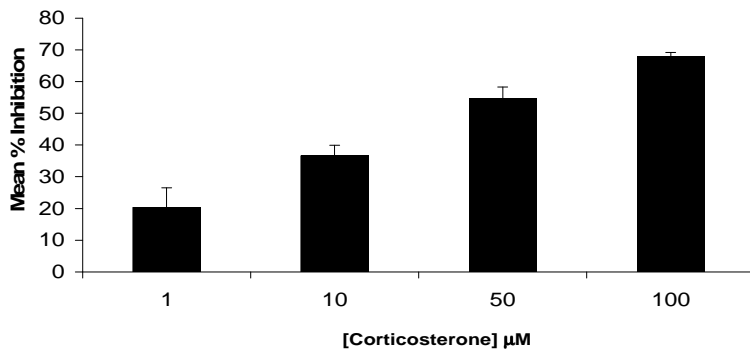


Figure 4.5 Concentration-dependent inhibition by a) testosterone, b) dihydrotestosterone and c) corticosterone of the formation of  $6\beta$ -naltrexol from naltrexone ( $30 \mu\text{M}$ ) in human liver cytosol (HLS# 11, 15 and 31). (Data represent mean  $\pm$  SD  $n=3$ ).

Table 4.4 Comparison of the sums of squares of competitive, non-competitive and non-competitive models for the calculation of the inhibition of 6 $\beta$ -naltrexol formation from naltrexone in human liver cytosol.

Inhibitor	Mean sum of squares (n=3)		
	Competitive	Non-Competitive	Uncompetitive
Naloxone	145	147	154
Menadione	31	30	39
Testosterone	17	22	29
Dihydrotestosterone	117	114	126
Corticosterone	33	39	52

Table 4.5  $K_i$  values for the inhibition of 6 $\beta$ -naltrexol formation from naltrexone in human liver cytosol preparations from 3 different livers, using a competitive inhibition model.

Inhibitor	$K_i$ ( $\mu$ M)
Naloxone	19.9 $\pm$ 5.3
Menadione	5.6 $\pm$ 2.8
Corticosterone	10.5 $\pm$ 4.7
Testosterone	0.28 $\pm$ 0.14
Dihydrotestosterone	0.73 $\pm$ 0.38

#### 4.5. Discussion

The assay continued to perform with a high degree of accuracy and precision at the lowest calibration concentration and all three QC values. This study showed that the hepatic enzymatic formation of 6 $\beta$ -naltrexol from naltrexone in human liver was confined to the cytosolic and not the microsomal fraction, exhibited considerable intersubject variability, and that the enzyme(s) involved could be inhibited by a number of compounds. The most potent inhibition was by the steroids testosterone, dihydrotestosterone and corticosterone, while the opioid antagonist naloxone also showed significant inhibition. The assay used in the current study to quantify 6 $\beta$ -naltrexol in human liver cytosol preparations was shown to be both simple, precise and accurate. Unlike assays of naltrexone and 6 $\beta$ -naltrexol in plasma and urine (Davidson et al., 1996; King et al., 1997), no extraction was necessary, and therefore an internal standard was not needed.



Using these human liver cytosol preparations there was considerable intersubject variability in both the  $V_{\max}$  (2.9-fold) and  $K_m$  (3.2-fold) values. This degree of variability has been shown in kinetic studies using enzymes from the cytochrome P450 family and the glutathione transferases (Ali, 2004). Intrinsic clearance ( $Cl_{int}$ ) values, which can be related to the *in vivo* intrinsic clearance suggest a drug of high hepatic extraction and showed an even larger variation (7.7-fold). Kume and co-workers demonstrated a variant allele of the AKR1C4 (DD4) enzyme (DD4<sub>(S145C/L311V)</sub>), with approximately one third the normal catalytic activity towards naltrexone (Kume et al., 1999); they suggested that this could account for the intersubject variabilities of the  $K_m$  and  $K_{cat}$  values found in an earlier study (Ohara et al., 1995). These differences in metabolism between individuals could result in large variability in plasma and brain naltrexone and 6 $\beta$ -naltrexol concentrations, and hence, could have an effect on drug efficacy, patient compliance and willingness to stay on naltrexone treatment.

The data obtained in the present study are consistent with ketone reductase involvement as menadione, a known inhibitor of ketone-reducing enzymes (Wermuth, 1981), significantly inhibited the formation of 6 $\beta$ -naltrexol. Additionally, 6 $\beta$ -naltrexol formation was not detected in the microsomal fraction previously shown to catalyse CYP450 reactions, under conditions used frequently in this laboratory for a number of substrates. The antipsychotic drug haloperidol is reduced *in vivo* by cytosolic ketone reductase to form the metabolite, reduced haloperidol (Inaba and Kovacs, 1989). This metabolite can be converted back to the parent compound by microsomal cytochrome P450 enzymes, resulting in recirculation of haloperidol (Tyndale et al., 1991). In the present study, there was no back-formation of 6 $\beta$ -naltrexol to naltrexone in the microsomal preparation tested. The conditions used were similar to those used by Pan and colleagues, who observed the CYP3A4 mediated formation of haloperidol from reduced haloperidol with a  $K_m$  of 51-59  $\mu$ M and a  $V_{\max}$  of 190-334 pmol/ mg/min (Pan et al., 1998).

The results obtained in the present are consistent with later work by Breyer-Pfaff and Nill, who showed that the predominant enzyme involved in the reduction of naltrexone to 6 $\beta$ -naltrexol is AKR1C4, with  $K_m$  values of 0.037 and 0.029 mM (n=2) (Breyer-Pfaff and Nill, 2004).

In order to further characterise the enzymes responsible for the formation of 6 $\beta$ -naltrexol from naltrexone, chemical inhibitor studies were conducted. The androgenic steroid testosterone, and its major metabolite dihydrotestosterone were very potent inhibitors, with mean  $K_i$  values

of 280 nM and 730 nM, respectively. However, finasteride, an inhibitor of 5 $\alpha$ -reductase, the enzyme responsible for the formation of dihydrotestosterone from testosterone *in vivo*, did not affect the formation of 6 $\beta$ -naltrexol. This is not surprising as 5 $\alpha$ -reductase is a microsomal enzyme. However, steroid hormones are metabolised by a number of different enzyme systems, both cytosolic and microsomal, including reductases, hydroxysteroid dehydrogenases and the cytochrome P450 mono-oxygenases (Schanzer, 1996). One such cytosolic enzyme (17 $\beta$ -hydroxysteroid dehydrogenase type I), has been shown to contain tyrosine and lysine residues which are found at the active sites of bacterial 3 $\alpha$ ,20 $\beta$  hydroxysteroid dehydrogenase, with similar secondary structural folds (Labrie et al., 1997). Although this enzyme is substantially more reactive towards oestradiol than testosterone (Blomquist et al., 1994), the dihydrodiol dehydrogenase responsible for the reduction of naltrexone to 6 $\beta$ -naltrexol may have sufficient homology with this and other forms of 17 $\beta$ -hydroxysteroid dehydrogenase to cause the potent inhibition by testosterone and dihydrotestosterone seen in the present study. Corticosterone, metabolised predominantly by adrenal microsomal 18-hydroxylase, was also a moderately potent inhibitor of 6 $\beta$ -naltrexol formation, but the significance of this is as yet unknown. The effects of other steroid compounds, including the components of oral contraceptive preparations and hormone replacement therapies are also unknown.

The inhibition by naloxone was not unexpected due to the similarity in the structures of naloxone (which also undergoes reduction) compared to naltrexone. Of the other compounds tested, in particular nicotine, the benzodiazepines (with the possible exception of diazepam), and other opioid compounds, there is little likelihood of drug/drug interactions *in vivo*, since their small degrees of inhibition at high concentration (100  $\mu$ M) would be predicted not to be clinically important since their circulating concentrations are lower. Haloperidol did not produce significant inhibition of 6 $\beta$ -naltrexol formation tested in the present study at 100  $\mu$ M, however this concentration is significantly lower than the reported  $K_m$  of haloperidol of 0.5-0.6 mM (Inaba and Kovacs, 1989).

The potent inhibition by steroids raises the possibility of variability in plasma 6 $\beta$ -naltrexol concentration due to variability in circulating steroid hormone concentrations. The  $K_i$  of testosterone in the present study was 280 nM, which is approximately 10-fold the upper limit of normal circulating plasma levels in males. Therefore, the clinical significance of this high degree of inhibition by the steroid hormones is unknown at this stage, but it is reasonable to

postulate that since these steroids are potent inhibitors of 6 $\beta$ -naltrexol formation, the reverse may also occur, and that long-term naltrexone use may have deleterious effects on steroid metabolism. Further work is needed to test this hypothesis, and the involvement of other steroid compounds that are present in plasma at relatively high concentrations.

In summary, the hepatic cytosolic formation of the pharmacologically active 6 $\beta$ -naltrexol showed wide interpatient variability, which could impact on naltrexone efficacy *in vivo*. The potent inhibition by androgenic steroids could cause further variability in naltrexone efficacy and contribute to adverse effects. Conversely, the effect of naltrexone treatment on levels of circulating steroid hormones in both males and females, and long-term studies in animals to assess any changes to steroidogenesis are warranted. The large partial hepatic intrinsic clearance is consistent with naltrexone having a high hepatic first pass when given orally. While naltrexone metabolism appears to be unaffected by the presence of likely co-administered drugs, the pharmacokinetic and pharmacogenetic factors affecting the formation and elimination of 6 $\beta$ -naltrexol could influence patients' willingness to remain on naltrexone treatment, and this needs to be investigated.

## 5. Pharmacokinetics of Naltrexone in Healthy Volunteers

### 5.1. Introduction

As reviewed in Chapter 1, when taken orally, in tablet or syrup form, naltrexone is rapidly and almost completely absorbed, is more than 95% metabolised (Kleber, 1985; Meyer et al., 1984), with very little of the unchanged drug (<2%) excreted in the urine (Cone et al., 1974; Meyer et al., 1984). As previously noted, the intersubject variation for pharmacokinetic parameters has been high in most studies.

Following intravenous (iv) doses of naltrexone (1 mg) in humans, the clearance values have been reported as being 5.15 l/min, a value which exceeds cardiac output (Wall, 1981). Apparent clearance was 5.70 l/min after an oral dose of 50 mg and 2.61 l/min following a subcutaneous dose of 5 mg (Wall, 1981; Wall et al., 1984), and an estimated value of 1.5 l/min was obtained by Meyer *et al* (1984) also after an oral dose of 50 mg. Following an oral dose of naltrexone of 50 mg, only 6.5% of the dose was excreted as unchanged drug in the faeces, indicating nearly complete absorption (Wall et al., 1981). Meyer *et al* (1984) calculated the oral bioavailability to be of the order of 5%, and Kogan *et al* (1977), obtained values in the order of 22%. Wall *et al* (1981) obtained a figure of 40% but calculations were not reported. There has also been a significant variation in the values reported for half-life of naltrexone. These have ranged from  $1.1 \pm 0.2$  hours (mean  $\pm$ SD) to  $10.3 \pm 3.3$  hours after single oral doses of 50 or 100 mg and 2.7 hours after an IV dose of one mg (Verebey et al., 1976; Wall et al., 1981);  $3.57 \pm 2.62$  hours following an oral dose of 50 mg (Meyer et al., 1984).

Similarly to half-life values, the data obtained for peak plasma concentrations also demonstrate high inter-subject variability. Verebey *et al* (1976) reported peak plasma naltrexone concentrations in humans approximately one hour after an oral dose of 100 mg, whereas the  $T_{max}$  (time taken to reach maximum plasma concentration) for 6 $\beta$ -naltrexol was two hours post-naltrexone. The first blood collection was at one hour post-dose in that study, and the second at two hours. In three of the four subjects, the one hour plasma concentration of naltrexone was higher than the two hour level, but the peak may have been earlier. Similarly for 6 $\beta$ -naltrexol, three of the four subjects had the highest concentrations at the two hour time-point, which were all higher than the four hour time point, but the peak plasma concentration could have been either side of the two hour point. In that study the mean (n=4)  $C_{max}$  values after an acute 100 mg oral dose of naltrexone were  $43.6 \pm 29.9$  ng/ml for naltrexone and  $87.2 \pm 25.0$  ng/ml for 6 $\beta$ -naltrexol. Both naltrexone and 6 $\beta$ -naltrexol were

detectable in the plasma 24 hours post-naltrexone administration, but there was no accumulation of naltrexone or the metabolite 6 $\beta$ -naltrexol with chronic dosing of 100 mg/day of naltrexone (Verebey et al., 1976). Meyer *et al* (1984) reported mean  $T_{max}$  values of  $0.95 \pm 0.39$  and  $0.91 \pm 0.37$  hours for naltrexone and 6 $\beta$ -naltrexol, respectively, with  $C_{max}$  concentrations of  $8.55 \pm 4.84$  and  $99.3 \pm 20.23$  ng/ml for the two compounds following a single oral dose of naltrexone in 24 healthy, young volunteers. Following a 100 mg oral dose of naltrexone in six subjects who had been maintained on long-term (11-29 months) treatment of 50 mg/day, Ferrari *et al*(1998), obtained values for  $T_{max}$  of  $73.3 \pm 37.3$  min for both naltrexone and 6 $\beta$ -naltrexol, with  $C_{max}$  (ng/ml) values of  $76 \pm 45$  and  $163 \pm 69$ , and  $t_{1/2}$  (hours) values of  $16 \pm 8$  and  $25 \pm 14$  for naltrexone and 6 $\beta$ -naltrexol, respectively.

This variance between different studies is thought not to be related to single or repeated administration, but could be related to assay specificity and sensitivity, different subject populations, or differences in the quantification methods and calculations of total drug or unchanged drug concentration (Gonzalez and Brogden, 1988).

Plasma binding of naltrexone has been reported as 20%, over a concentration range of 0.1-500 ng/ml and was species independent (Ludden et al., 1976). The apparent volume of distribution ( $V_{\beta}$ ) calculated as  $f \cdot D / AUC_{\beta}$  (where  $f$  is the systemic availability,  $D$  is the administered dose, and  $AUC$  is the area under the plasma level curve) has been reported as 16.1 l/kg after a single dose and 14.2 l/kg after repeated oral doses of 100 mg (Kogan et al., 1977).

Few researchers have examined the concentrations of naltrexone or 6 $\beta$ -naltrexol in saliva following administration of naltrexone. Saliva is a non-invasive, easily collectable biological fluid and as such could be useful in the detection and quantification of many compounds. Verebey *et al* (1980) examined the differential distribution of naltrexone, 6 $\beta$ -naltrexol and 2-hydroxy-3-methoxy 6 $\beta$ -naltrexol (HMN) in plasma, red blood cells, saliva and urine of four volunteers after 2x200 mg doses of naltrexone administered 16 and 24 hours prior to collection. The levels obtained for saliva and plasma were similar, with mean naltrexone concentrations of  $9.4 \pm 5.2$  and  $13.9 \pm 17.1$  ng/ml for plasma and saliva, respectively. The plasma 6 $\beta$ -naltrexol concentrations were higher than naltrexone, but similar in plasma and saliva, being  $200.3 \pm 39.1$ , and  $166.7 \pm 184$  ng/ml, respectively. The authors concluded that measuring 6 $\beta$ -naltrexol in plasma, saliva or urine, or HMN in red blood cells, could more

efficiently monitor naltrexone status of the body than measuring naltrexone itself in any body fluids.

Previously, there have been reports of various adverse reactions to naltrexone. These have included nausea, dizziness and spontaneous erection. In one study of 24 male social drinkers, those who reported one or more subjective side effects such as headache, anxiety or erection, had significantly higher urinary concentrations of 6 $\beta$ -naltrexol than those in whom these effects were absent (King et al., 1997). As the plasma concentrations were not analysed in this study, it is not possible to correlate these findings with plasma levels of the naltrexone parent or metabolites, and the significance of the urinary finding is unknown.

## **5.2. Aim**

The aim of the current study was to determine the pharmacokinetics and bioavailability of naltrexone in healthy volunteers and to assess the secretion of naltrexone and 6 $\beta$ -naltrexol in saliva after oral and IV administration. This was done using a specific and sensitive HPLC with electrochemical detection assay, capable of simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol. Additionally, the subjective side effects experienced as a result of naltrexone administration were to be assessed in relation to the blood, urine and saliva concentrations of naltrexone and 6 $\beta$ -naltrexol.

## **5.3. Methods**

### **5.3.1. Drugs**

The naltrexone for oral administration (50 mg tablets of naltrexone hydrochloride marketed as ReVia) was obtained from Orphan Australia Pty Ltd, (Berwick, Australia). The naltrexone hydrochloride used for the intravenous administration was a kind gift from Dr Sandor Hosztafi (ICN Alkaloida Co, Budapest, Hungary). Solutions of one mg/ml naltrexone HCl in saline for injection were prepared by the Pharmacy Department, Royal Adelaide Hospital. Therefore, the tablets contained 45.2 mg of naltrexone base and the IV solution contained 902  $\mu$ g of naltrexone base. The purity of the tablets and powder for injection was verified by Dr. Simon Pyke, Department of Organic Chemistry, University of Adelaide using mass spectrometry. All other chemicals used in the HPLC analyses of naltrexone and 6 $\beta$ -naltrexol have been described previously (see 3.3).

### **5.3.2. Volunteers**

Ethics approval for the following study was obtained from the Royal Adelaide Hospital Research Ethics Committee (Protocol No:000825). The inclusion criteria were: male,  
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healthy, aged between 18 and 45, with body weights between 55 and 80 kg or within 20% of ideal for range and height. The exclusion criteria were: any regular medication in the previous month; any indication of drug abuse, including cannabinoids, opioids and benzodiazepines; consumption of alcohol at levels above National Health and Medical Research Council guidelines (>40 g ethanol/day for males); any volunteer who by virtue of present or past history, or on the basis of physical examination was deemed to be unsuitable or unfit for inclusion in this study. Particular reference was made to: a) hepatic (abnormal serum biochemistry- total bilirubin >1.5 times, alkaline phosphatase (ALP) >1.2 times, alanine aminotransferase (ALT) >1.5 times and  $\gamma$ -glutamyltransferase (GGT) >2.0 times, the upper limit of normal ranges; albumin <34 or >58 g/L), renal (calculated creatinine clearance <70 ml/min) or any gastrointestinal disease; b) positive results for HIV, Hepatitis B or C surface antigen; c) a history of allergy to opioids. Further exclusion criteria included a blood bank or other blood donation (>300 ml) within the previous three months.

Six healthy adult males were recruited for the study. All volunteers undertook a physical examination (including medical history) and provided samples for laboratory evaluation including biochemistry, haematology, virology (HIV and Hepatitis C antibodies, and Hepatitis B antigen) and urinalysis for drugs of abuse (including benzodiazepines, opiates, methadone, sympathomimetic amines, cannabinoids, barbiturates, cocaine, dextropropoxyphene, phencyclidine and ethanol) prior to the study, and all provided informed written consent. The mean age for the volunteers was  $23.3 \pm 5.0$  (range 19-33) years, with a mean weight of  $80.2 \pm 8.9$  (range 71-95) kg. All were non-smokers (not intended as part of the inclusion/exclusion criteria). The patient demographics and laboratory results are shown in Table 5.1 below.

### **5.3.3. Experimental Design and Protocol**

The protocol involved a randomised, crossover design whereby the six volunteers received either oral (one 50 mg tablet with 200 ml water) or intravenous (1 mg bolus administered over 30 seconds) naltrexone followed by the alternative treatment at least one week later. The naltrexone oral dose of 50 mg, and intravenous dose of 1 mg were chosen as both had been used previously in pharmacokinetic studies, and were shown to be well tolerated, and were expected to produce quantifiable levels of parent and metabolite in body fluids. For both studies the subjects fasted overnight (> 10 hour) and presented to the designated ward of the Royal Adelaide Hospital at 7.30 am. An indwelling cannula with an extension set and a three-way tap (Baxter Health Care Corp, Ill. USA) was inserted into a forearm vein by a medical

officer prior to administration of any drug treatment. The cannula site was covered with a clear sterile dressing (Smith and Nephew Ltd, Middlesex, England). Blood samples were obtained via the three-way tap by withdrawing and discarding 2 ml blood prior to each sample and flushing with 2 ml physiological saline (0.9% sodium chloride) for injection after each sample. The IV dose was administered into the forearm vein of the arm without the catheter. The cannula remained in place for 16 hours where possible at which time the volunteer left the ward, and subsequent samples were obtained via venepuncture. Volunteers returned to the Royal Adelaide Hospital for further blood samples by venepuncture as required.

### **5.3.3.1. Biological Fluid Sampling**

Blood samples were collected prior to naltrexone administration and then at 0.08, 0.17 0.33 (IV only), 0.25 (oral only) 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3, 4, 5, 6, 8, 10, 12, 16, 24, 30, 48 and 60 hr post-naltrexone administration. The blood samples were centrifuged for 10 min at 3500 rpm, and the plasma was stored at -20 °C until analysis. Additional samples were collected at 0.5 and 6 hours post-naltrexone administration (oral and iv) which were not centrifuged, for whole blood analyses.

Non-stimulated saliva samples were collected at the same time as all blood samples as listed above. The pH of the saliva samples was recorded and the samples were stored at -20 °C until analysis.

Urine was collected prior to naltrexone administration and then all urine was collected in aliquots of 0-3, 3-6, 6-12, 12-24, 24-36, 36-48, 48-60, 60-72, 72-84 and 84-96 hr post-naltrexone after both oral and IV administration. Volume and pH were measured on the urine samples, which were then aliquoted and stored at -20 °C until analysis. All samples (except urines collected after 12 hr) were placed in a freezer within 20 minutes of collection.

### **5.3.3.2. Pharmacodynamic Measurements**

Subjective naltrexone-related responses were assessed using the Profile of Mood States (McNair et al., 1971) and the Methadone Symptoms Checklist (Dyer and White, 1997). The volunteers completed these questionnaires prior to and 1, 3, 6, 12, 24 and 48 hr post-naltrexone.

The Profile of Mood States (POMS) questionnaire consists of 65 questions, which can be divided into six subscales of Tension/Anxiety, Depression/Dejection, Anger/Hostility, Vigour/Activity, Fatigue/Inertia and Confusion/Bewilderment. The answers were scored on an analogue scale of 0 (Not at all) to 4 (Extremely). The total mood score was calculated as



the Vigour/Activity score subtracted from the sum of the individual negative mood states of Depression/Dejection, Anger/Hostility, Fatigue/Inertia and Confusion/Bewilderment.

The Methadone Symptom Checklist (MSC) was designed to assess opioid withdrawal severity in methadone-maintenance patients (Dyer and White, 1997). This was further modified to address only those symptoms that had been previously reported following naltrexone treatment in healthy volunteers. A five-category scale, ranging between 'none' and 'extreme' was used to measure the severity of specific symptoms such as nausea, dry mouth, stomach cramps and confusion. The symptoms were scored on the basis of their presence (1) or absence (0). This checklist was used solely to assess the effect of naltrexone, as all volunteers were opioid naïve.

### **5.3.4. Analyses of biofluids and calculations**

#### **5.3.4.1. Plasma, Urine and Saliva analyses**

Samples were analysed for naltrexone and 6 $\beta$ -naltrexol using HPLC with electrochemical detection. The assay conditions were as described previously in 3.5.2 (plasma), 3.6.1 (urine), 3.8.2 (saliva) and 3.9.2 (whole blood). These assays were validated as described in Chapter 3. The ongoing performance of the assays was assessed using three quality control samples in duplicate, and each assay had to fulfil acceptance criteria of  $r^2 > 0.99$  for the calibration curve, at least 4 of the 6 quality control values and 6 of the 8 calibration concentrations within 20% of the nominal value.

#### **5.3.4.2. Plasma protein binding**

The plasma protein binding of naltrexone and 6 $\beta$ -naltrexol was calculated by comparison of concentrations in plasma and plasma ultrafiltrates. The ultrafiltered plasma samples were prepared and assayed as described in 3.7.2. The samples used were those associated with the maximum concentration of naltrexone following the 50 mg oral treatment.

#### **5.3.4.3. Blood:Plasma ratio calculations**

Blood:plasma concentration ratios were calculated by dividing the concentrations of analytes obtained in the whole blood samples (corrected for packed cell volume) by the concentrations in the corresponding plasma samples.

#### **5.3.4.4. Calculation of analyte conjugates in urine**

In order to quantify the conjugated naltrexone and 6 $\beta$ -naltrexol in pooled urine samples, the

urine samples were treated with  $\beta$ -glucuronidase (from *H-pomatia*) diluted from 330 000 U/g purchased from Sigma Aldrich (St Louis, Mo, USA). The samples were treated with 2500, 5000, 10000, 15000 and 20000 U/ml  $\beta$ -glucuronidase to obtain the concentration required for the assay. No significant differences in urine concentrations of naltrexone and 6 $\beta$ -naltrexol were obtained with the different  $\beta$ -glucuronidase concentrations, therefore, 10000 U/ml was used as this concentration had been used previously in this laboratory (Chen et al., 1990). Aliquots of 100  $\mu$ l of the pooled urine samples were treated with 100  $\mu$ l of  $\beta$ -glucuronidase (to give a final concentration of 10000 U/ml) and 800  $\mu$ l water. These samples were shaken for 16 hours at 37 °C, then 100  $\mu$ l internal standard was added and the samples were extracted as described above.

The concentrations of the conjugated analytes were calculated by subtracting the analyte concentrations of the unhydrolysed samples from the hydrolysed urine sample excretions.

### 5.3.5. Pharmacokinetic and Statistical Analyses

The following parameters were derived or calculated where possible: a) IV naltrexone-AUC, total body clearance, renal clearance, half-life, partial clearance to 6 $\beta$ -naltrexol and b) oral naltrexone- $T_{max}$ ,  $C_{max}$  and bioavailability. The  $T_{max}$  and  $C_{max}$  values were obtained by visual examination of the data. The renal clearance and total per cent of the naltrexone dose excreted were calculated for 6 $\beta$ -naltrexol. The parameters were calculated as shown below:

$$CL = \frac{D_{IV}}{AUC_{IV}}$$

$$F = \frac{AUC_{ORAL}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{oral}}$$

$$CL_R = fe * CL$$

$$CL_{to\ 6\beta\text{-naltrexol}} = (De_{6\beta\text{-naltrexol}}/AUC_{naltrexone})/60(min)$$

$$CL_B = CL/(B:P)$$

Where D is dose, De is the amount of 6 $\beta$ -naltrexol in naltrexone equivalents excreted in urine, CL is clearance, AUC is the area under the plasma concentration versus time curve  
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(calculated using the trapezoidal rule),  $F$  is the bioavailability and  $f_e$  the fraction of the IV dose recovered in urine as naltrexone.  $CL_B$  is the clearance from blood, B:P is the blood to plasma ratio. The terminal elimination half-life ( $t_{1/2}$ ) was determined by linear regression of the terminal log-concentration data points after plotting the plasma concentrations against time. The  $AUC_{0-96hr}$  of saliva and plasma were compared using paired Student's T-tests. The saliva:plasma concentration ratios were also calculated. The effect of pH on saliva concentrations of naltrexone and 6 $\beta$ -naltrexol was analysed by linear regression. The blood:plasma concentration ratios for oral and IV administration were compared using One-way ANOVA to compare all columns, using GraphPad Prism Software (GraphPad Software Inc, San Diego, CA, USA). The drug administration (IV vs oral), and time effects (0-48 hours) for the POMS and MSC questionnaires were analysed statistically using covariate ANOVA (SPSS V10.1 for Windows).

## **5.4. Results**

### **5.4.1. Patient demographics**

Table 5.1 below shows the demographic characteristics and laboratory results for the six volunteers who participated in the pharmacokinetic study. All volunteers fulfilled the inclusion criteria, and no volunteer had any clinically significant abnormalities of any of the parameters tested. Therefore, the study was conducted on six healthy males, five Caucasian and one Asian, aged between 19 and 33 years, with no history or evidence of drug or alcohol abuse.

Table 5.1 Demographics and laboratory results for the volunteers. RI refers to the appropriate reference interval.

All subjects were Caucasians except #(Asian)

Parameter (units) [RI]	Subjects					
	01	02	03	04#	05	06
Sex	M	M	M	M	M	M
Age (years)	24	22	21	33	21	19
Height (cm)	187	182	180	175	176	180
Weight (kg)	95	86	78	73	71	78
Pulse (beats/minute)	73	65	80	80	60	100
Respiratory Rate (breaths/min)	15	18	16	14	16	14
BP-Systolic (mmHg)	150	130	125	125	125	130
BP-Diastolic (mmHg)	90	40	60	90	70	60
Hb (g/L) [135-180]	169	151	138	154	144	144
WCC (x10 <sup>9</sup> /L) [4.0-11.0]	3.65	6.62	4.97	7.31	7.76	9.4
Platelets (10 <sup>9</sup> /L) [150-400]	199	262	251	244	258	292
Sodium (mM) [137-145]	143	144	144	141	145	141
Potassium (mM) [3.1-4.2]	4.2	3.8	3.8	3.5	3.3	3.7
Chloride (mM) [100-109]	99	101	104	98	105	99
Bicarb (mM) [22-32]	26	26	25	26	26	25
Glucose (mM) [4.2-5.5]	4.4	4.2	5.5	5.1	5.1	4.9
Urea (mM) [2. 7-7.2]	4.5	3.8	4.5	3.7	4.4	3.7
Creatinine (mM) [0.050-0.120]	0.092	0.083	0.073	0.077	0.096	0.08
Urate (mM) [0.25-0.5]	0.43	0.29	0.26	0.4	0.31	0.23
Phosphate (mM) [0.80-1.45]	1.2	1.4	1.12	0.94	1.05	1.05
Calcium (mM) [2.10-2.55]	2.33	2.31	2.29	2.32	2.27	2.3
Albumin (g/L) [34-48]	39	48	45	44	44	45
Globulins (g/L) [22-35]	35	39	31	39	33	42
Total Bilirubin (mM) [6-24]	35	8	9	17	12	14
GGT (U/L) [0-60]	50	13	14	28	20	8
ALP (U/L) [30-110]	68	102	46	99	120	125
ALT (U/L) [0-55]	46	20	19	34	16	12
AST (U/L) [0-45]	44	32	27	31	30	29
LDH (U/L) [110-230]	216	157	175	159	159	158

BP=blood pressure, Hb=haemoglobin, WCC=white cell count, GGT= $\gamma$ -glutamyl transferase, ALP=alkaline phosphatase, ALT=alanine amino transferase, AST=aspartate amino transferase, LDH=lactate dehydrogenase.

## 5.4.2. Ongoing performance of the assays

### 5.4.2.1. Plasma assay

The validation of the assay for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in human plasma is described in Chapter 3 (3.3). Based on the results of the quality control samples, the plasma assays for naltrexone and 6 $\beta$ -naltrexol continued to perform with a high degree of accuracy (>85% naltrexone, >94% 6 $\beta$ -naltrexol) and precision (coefficient of variation <10% for both analytes), as shown in Table 5.2.

Table 5.2 Ongoing accuracy and precision of QC values for naltrexone and 6 $\beta$ -naltrexol in plasma. LQC, MQC and HQC represent the low, medium and high quality controls, respectively.

Plasma	n	Naltrexone			6 $\beta$ -naltrexol		
		Nominal Conc. (ng/ml)	Mean Accuracy %	Precision % CV	Nominal Conc. (ng/ml)	Mean Accuracy %	Precision % CV
LQC	14	1.75	86	6.6	7.5	96	9.4
MQC	14	7.5	90	9.7	37.5	94	7.4
HQC	14	17.5	90	4.6	75	97	4.8

### 5.4.2.2. Urine assay

The validation of the assay for the simultaneous detection of naltrexone and 6 $\beta$ -naltrexol in urine is described in Chapter 3 (3.4). The assay of naltrexone (0.5-25 ng/ml) and 6 $\beta$ -naltrexol (2-100 ng/ml) continued to give high accuracy (>90% naltrexone; >98% 6 $\beta$ -naltrexol) and precision (CV<11%) for all quality control samples tested as shown in Table 5.3. In order to quantify both naltrexone and 6 $\beta$ -naltrexol in  $\beta$ -glucuronidase-hydrolysed samples and 6 $\beta$ -naltrexol in some of the unhydrolysed samples, calibration curves of naltrexone (5, 10, 20, 40, 60, 80 and 100 ng/ml) and 6 $\beta$ -naltrexol (100, 250, 500, 750, 1000, 1500, 1750 and 2000 ng/ml) were constructed. These assays were not performed simultaneously as the 6 $\beta$ -naltrexol detector sensitivity required adjustment as described in Chapter 3. The ongoing performance of the high-concentration assays is reported in Table 5.4 and shows accuracy values of >94% and precision values of >94% for both naltrexone and 6 $\beta$ -naltrexol.

Table 5.3 Ongoing accuracy and precision of QC samples for naltrexone and 6 $\beta$ -naltrexol in urine. LQC, MQC and HQC represent the low, medium and high quality controls, respectively.

Urine	Naltrexone				6 $\beta$ -naltrexol			
	n	Nominal Conc. (ng/ml)	Mean Accuracy %	Precision % CV	n	Nominal Conc. (ng/ml)	Mean Accuracy %	Precision % CV
LQC	14	1.75	99	10.9	12	7.5	101	9.0
MQC	18	7.5	96	8.1	15	37.5	98	6.3
HQC	18	17.5	91	5.1	15	75	100	4.7

Table 5.4 Ongoing accuracy and precision of high concentration QC values for naltrexone and 6 $\beta$ -naltrexol in urine. Note: Assays not performed simultaneously. LQC, MQC and HQC represent the low, medium and high quality controls, respectively.

Urine	Naltrexone				6 $\beta$ -naltrexol			
	n	Nominal Conc. (ng/ml)	Mean Accuracy %	Precision % CV	n	Nominal Conc. (ng/ml)	Mean Accuracy %	Precision % CV
LQC	9	15	103	3.2	15	350	98	3.2
MQC	10	50	106	1.9	16	700	96	5.6
HQC	9	75	104	1.9	14	1250	104	5.3

#### 5.4.2.3. Saliva assay

The ongoing performance data for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in human saliva are shown in Table 5.5, with the accuracy of the quality control samples being >82% for naltrexone and >94% for 6 $\beta$ -naltrexol, with coefficients of variation of <12% for both analytes.

Table 5.5 Ongoing accuracy and precision of QC values for naltrexone and 6 $\beta$ -naltrexol in saliva. LQC, MQC and HQC represent the low, medium and high quality controls, respectively.

Saliva	Naltrexone				6 $\beta$ -naltrexol			
	n	Nominal Conc. (ng/ml)	Mean Accuracy %	Precision % CV	n	Nominal Conc. (ng/ml)	Mean Accuracy %	Precision % CV
LQC	15	1.75	83	6.2	15	7.5	95	7.1
MQC	16	7.5	91	12	16	37.5	102	8.6
HQC	14	17.5	82	4.7	16	75	100	8.5

#### 5.4.2.4. Combined results

Table 5.6 shows the means, standard deviations and %CV of slopes and intercepts for the calibration curves of naltrexone and 6 $\beta$ -naltrexol in plasma, urine and saliva. The slopes (with the exception of plasma naltrexone) remained relatively stable, with CV's of <15%.

Table 5.6 Mean, SD and %CV of slopes and intercepts for the assays of naltrexone and 6 $\beta$ -naltrexol in human fluids. Urine Low is the calibration curve 2-100 ng/ml; Urine High is the calibration curve 100-2000 ng/ml. n=7 for all assays except urine low, (n=6).

	Naltrexone			6 $\beta$ -Naltrexol			
	Plasma	Saliva	Urine	Plasma	Saliva	Urine Low	Urine High
<b>Slope</b>							
Mean	0.0780	0.0772	0.0849	0.0879	0.0617	0.0736	0.0087
SD	0.013	0.0041	0.0109	0.0095	0.0041	0.0045	0.0006
%CV	16.7	5.3	12.8	10.8	5.3	6.1	6.6
<b>Intercept</b>							
Mean	0.0034	-0.0088	-0.0071	-0.0403	-0.0111	0.0385	-0.1622
SD	0.0264	0.0049	0.071	0.0772	0.0049	0.0339	0.0611
<b>Corr coeff r<sup>2</sup></b>							
Mean	0.9952	0.9978	0.9932	0.9964	0.9969	0.9928	0.9944
SD	0.0027	0.0022	0.0024	0.0023	0.0022	0.0014	0.0015
%CV	0.27	0.22	0.24	0.23	0.22	0.14	0.15

### 5.4.3. Pharmacokinetics of Intravenous Naltrexone Administration

#### 5.4.3.1. Plasma Data

Following the intravenous dose of naltrexone (1 mg of naltrexone hydrochloride), plasma concentrations were below the limit of quantification of 2.0 ng/ml for 6 $\beta$ -naltrexol for all samples and low for naltrexone (being unquantifiable in 5/6 of the volunteers after 12 hours). Therefore, parameters such  $C_{max}$ ,  $T_{max}$  and AUC could not be calculated for 6 $\beta$ -naltrexol and half-life could not be calculated for naltrexone, as there were too few data points in a terminal log-linear phase. As naltrexone has been shown to have a late terminal phase, (which could not be measured in this study), the data below may indicate and underestimation of AUC, and hence an overestimation of plasma clearance. The individual plasma profiles for naltrexone are shown in Figure 5.1 for up to 8 hours post-iv (1 mg) administration. Table 5.7 shows the pharmacokinetic data for naltrexone, with the mean ( $\pm$ SD) values for these parameters, excluding volunteer #6 who had a calculated urinary excretion greater than the administered dose are shown in Table 5.8

Table 5.7 Pharmacokinetic parameters for naltrexone following intravenous naltrexone (1mg). Note: (n=5). Subject #6 data excluded from mean and standard deviation (SD) calculations.

Subject	$C_{max}$ (ng/ml)	$T_{max}$ (hr)	AUC (ng/ml/hr)	$CL_R$ (ml/min)	CL to 6 $\beta$ -nol (ml/min)	CL (ml/min)	$CL_B$ (ml/min)
1	3.05	0.08	0.72	486	9486	23155	12316
2	3.00	0.08	4.72	7	1796	3582	2357
3	3.66	0.08	1.92	191	3298	8683	3727
4	2.95	0.08	1.19	266	4385	14010	-
5	3.56	0.08	2.47	331	2637	6750	5720
6	2.08	0.17	6.71	42	4438	2485	-
<b>Mean</b>	<b>3.24</b>	<b>0.08</b>	<b>2.2</b>	<b>256</b>	<b>4320</b>	<b>11226</b>	<b>6030</b>
<b>SD</b>	<b>0.34</b>	<b>0.00</b>	<b>1.6</b>	<b>177</b>	<b>3039</b>	<b>7677</b>	<b>4412</b>



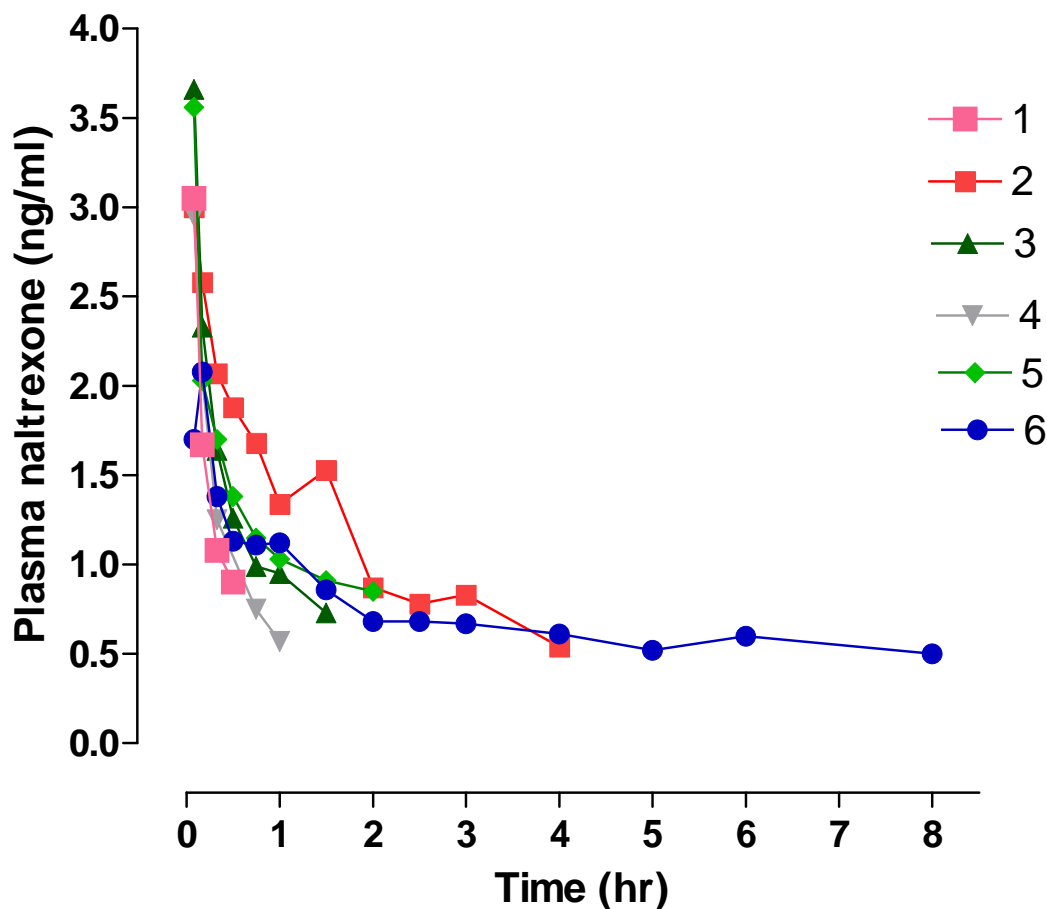


Figure 5.3 Individual plasma naltrexone profiles for the first 8 hours after intravenous administration (1mg) in six healthy volunteers.

#### 5.4.3.2. Urine Data

Table 5.8 shows the individual and mean ( $\pm$ SD) excretion data for the six volunteers for both the unhydrolysed and the  $\beta$ -glucuronide hydrolysed samples, for naltrexone and  $6\beta$ -naltrexol. The urine samples collected over 24 hours were pooled for the hydrolysis. In one volunteer, unconjugated naltrexone was detectable up to 24 hours (the volunteer who excreted > 1mg), one volunteer had quantifiable naltrexone in the 12-hour samples, three had detectable levels to six hours, and the remaining volunteer had no quantifiable naltrexone after three hours. Naltrexone was excreted mostly as the glucuronide conjugate (95%) whereas  $6\beta$ -naltrexol was excreted mainly in the unconjugated form (67.5%).

Table 5.8 Data for urinary excretion (0-96 hours) of naltrexone and 6 $\beta$ -naltrexol (% of naltrexone dose excreted) in six volunteers after an IV (1mg) dose of naltrexone. Mean  $\pm$  SD excludes volunteer #6

	Naltrexone			6 $\beta$ -naltrexol			Total % Dose Excreted
	Uncon- jugated	Conjugated	Total	Uncon- jugated	Conjugated	Total	
1	2.1	11.8	13.9	30.0	11.1	41.1	55.0
2	0.2	14.0	14.2	43.3	7.7	51.0	65.2
3	2.2	10.2	12.4	23.6	14.5	38.1	50.5
4	1.9	15.3	17.2	17.0	14.4	31.4	48.6
5	4.9	11.4	16.3	26.2	13.0	39.2	55.5
6	1.7	58.8	60.5	129.6	49.6	179.2	259.7
<b>Mean</b>	<b>2.26</b>	<b>12.5</b>	<b>14.8</b>	<b>28.0</b>	<b>12.1</b>	<b>40.2</b>	<b>55.0</b>
<b>SD</b>	<b>1.7</b>	<b>2.1</b>	<b>2.0</b>	<b>9.8</b>	<b>2.8</b>	<b>7.1</b>	<b>2.88</b>

#### 5.4.3.3. Saliva Data

Naltrexone and 6 $\beta$ -naltrexol were below the limits of quantification (0.5 ng/ml naltrexone, 2.0 ng/ml 6 $\beta$ -naltrexol) in saliva following the intravenous dose of naltrexone in all samples collected.

#### 5.4.4. Pharmacokinetics of Oral Naltrexone Administration

##### 5.4.4.1. Plasma Data

Table 5.9 shows the individual and mean ( $\pm$ SD) data for naltrexone plasma pharmacokinetic data for the six volunteers following an oral dose of naltrexone (50 mg). Table 5.10 shows the corresponding data for 6 $\beta$ -naltrexol. Figure 5.2 shows the individual plasma concentration versus time profiles for naltrexone and 6 $\beta$ -naltrexol as semi-log plots. While blood samples were collected for 96 hours following naltrexone administration, naltrexone was quantifiable in the plasma in only 2 volunteers after eight hours and in only one volunteer after 12 hours. The 6 $\beta$ -naltrexol was undetectable in all volunteers 60 hours post-naltrexone. Maximum plasma concentrations of both the parent drug and the metabolite were achieved within one hour of administration, with the concentrations ranging from 1-20 ng/ml for naltrexone, and 77-149 ng/ml for 6 $\beta$ -naltrexol. The mean elimination half-life from plasma for naltrexone was  $1.88 \pm 0.47$  hours and for 6 $\beta$ -naltrexol  $13.3 \pm 2.6$  hours. The values ranged from 1.45 to 2.71 hours for naltrexone, and 10.5 to 16.1 hours for 6 $\beta$ -naltrexol. The coefficient ( $r^2$ ) of the

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regression equation for the determination of elimination half-life was greater than 0.99 for 6 $\beta$ -naltrexol, while for naltrexone the  $r^2$  was greater than 0.97 for all volunteers.

Table 5.9 Pharmacokinetic parameters for naltrexone for each of the volunteers following oral naltrexone administration (50 mg). 6 $\beta$ -nol is 6 $\beta$ -naltrexol.

	<b>C<sub>max</sub></b>	<b>T<sub>max</sub></b>	<b>AUC</b>	<b>t<sub>1/2</sub></b>	<b>F</b>	<b>CL<sub>R</sub></b>	<b>CL to 6<math>\beta</math>-nol</b>
	<b>(ng/ml)</b>	<b>(hr)</b>	<b>(ng.hr/ml)</b>	<b>(hr)</b>		<b>(ml/min)</b>	<b>(ml/min)</b>
1	9.6	1.0	18.5	1.5	0.51	103	16990
2	3.3	0.5	17.4	2.7	0.07	79	22368
3	10.9	0.5	18.2	1.5	0.19	14	13576
4	19.4	0.5	37.9	2.0	0.64	106	5606
5	1.1	0.75	15.9	1.8	0.13	106	16275
6	3.9	1.0	12.8	1.8	0.04	57	21316
<b>Mean</b>	<b>8.03</b>	<b>0.71</b>	<b>20.1</b>	<b>1.9</b>	<b>0.26</b>	<b>77.5</b>	<b>16022</b>
<b>SD</b>	<b>6.7</b>	<b>0.25</b>	<b>9.0</b>	<b>0.45</b>	<b>0.25</b>	<b>36.7</b>	<b>6063</b>

Table 5.10 Pharmacokinetic parameters for 6 $\beta$ -naltrexol for each of the volunteers following oral naltrexone administration (50 mg).

	<b>C<sub>max</sub></b>	<b>T<sub>max</sub></b>	<b>AUC</b>	<b>t<sub>1/2</sub></b>	<b>CL<sub>R</sub></b>
	<b>(ng/ml)</b>	<b>(hr)</b>	<b>(ng.hr/ml)</b>	<b>(hr)</b>	<b>(ml/min)</b>
1	95	1.0	525	14.9	600
2	149	0.75	821	16.1	474
3	147	0.75	598	10.5	413
4	77	0.75	629	12.0	339
5	173	0.75	891	15.8	291
6	122	1.0	675	10.6	405
<b>Mean</b>	<b>127</b>	<b>0.83</b>	<b>690</b>	<b>13.3</b>	<b>420</b>
<b>SD</b>	<b>36</b>	<b>0.13</b>	<b>139</b>	<b>2.6</b>	<b>108</b>

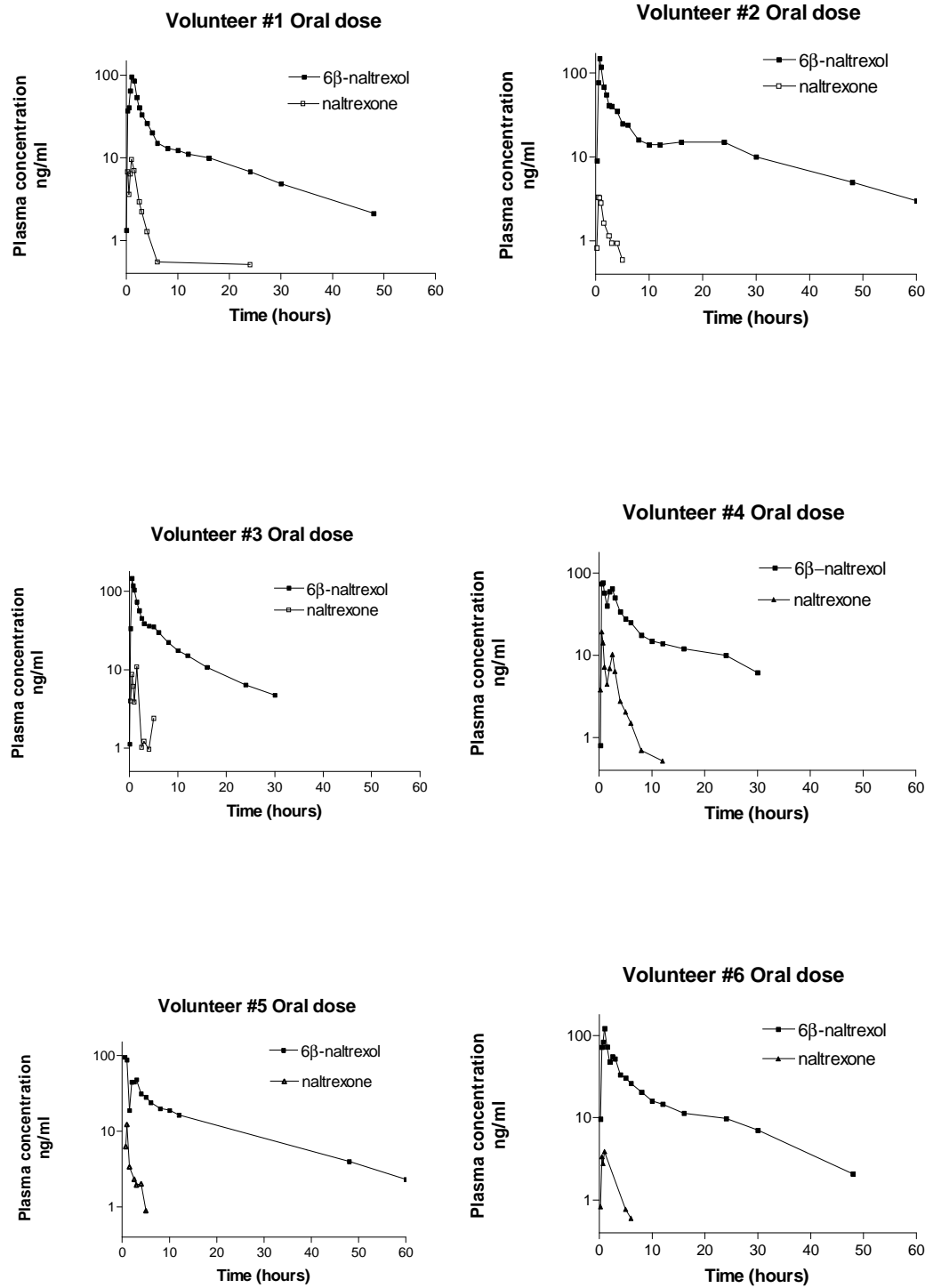


Figure 5.4 Individual plasma concentration versus time profiles of naltrexone and 6β-naltrexol for the six volunteers following an oral dose (50 mg) of naltrexone. Note, semi-log scale.

**5.4.4.2. Urine data**

In accordance with the IV dose, following an oral dose of naltrexone, the majority of the drug was excreted as the metabolite 6 $\beta$ -naltrexol. The mean urinary excretion data, as shown in Table 5.11 were  $2.8 \pm 1.1\%$  of the dose excreted as conjugated plus unconjugated naltrexone with a range of 2.1-5.0%. A mean of  $32 \pm 7.6\%$  with a range of 23-45 of the dose was excreted as conjugated plus unconjugated 6 $\beta$ -naltrexol. Only  $35.4 \pm 6.2\%$  of the total dose was recovered in the urine. The majority of the naltrexone excreted was in the glucuronide conjugated form (96%), while 6 $\beta$ -naltrexol was mainly excreted unconjugated (69%).

Table 5.11 Mean  $\pm$  SD urinary excretion of conjugated and unconjugated naltrexone and 6 $\beta$ -naltrexol following oral naltrexone administration (50 mg) expressed as a per cent of the naltrexone dose (n=6).

	Naltrexone			6 $\beta$ -naltrexol			Total % Dose Excreted
	Uncon- jugated	Conjugated	Total	Uncon- jugated	Conjugated	Total	
1	0.23	2.21	2.44	22.3	13.3	35.6	38.04
2	0.16	2.24	2.40	33.4	11.4	44.8	47.20
3	0.03	2.62	2.65	23.0	10.1	33.1	36.65
4	0.48	4.53	5.01	15.7	7.1	22.8	27.81
5	0.20	1.89	2.09	17.5	9.5	27.0	29.09
6	0.09	2.21	2.30	22.5	8.9	31.2	33.50
<b>Mean</b>	<b>0.20</b>	<b>2.62</b>	<b>2.82</b>	<b>22.4</b>	<b>10.1</b>	<b>32.4</b>	<b>35.38</b>
<b>SD</b>	<b>0.16</b>	<b>0.97</b>	<b>1.09</b>	<b>6.2</b>	<b>2.1</b>	<b>7.6</b>	<b>7.05</b>

**5.4.4.3. Saliva Data**

The mean ( $\pm$  SEM) naltrexone and 6 $\beta$ -naltrexol saliva concentration versus time profiles are shown in Figures 5.5 and 5.6, respectively. Tables 5.12 and 5.13 show the individual concentrations at each time point of naltrexone and 6 $\beta$ -naltrexol for the six volunteers following an oral (50 mg) dose of naltrexone. For naltrexone, the maximum concentration was obtained at 15 minutes post-dose for all volunteers except #3, who had the highest naltrexone concentration (249 ng/ml). While the volunteers rinsed their mouths and drank water following the oral dose of naltrexone, this result could be a result of naltrexone contamination of the saliva sample. The peak naltrexone concentrations obtained ranged from 74-249 ng/ml. Volunteer #3 also had the highest 6 $\beta$ -naltrexol concentration of 455 ng/ml

obtained in the shortest time after the naltrexone dose (45 minutes). The elimination half-life values for naltrexone ranged from 2.5 to 3.3 hours, with a mean value of  $2.9 \pm 0.3$  hours.

The peak  $6\beta$ -naltrexol concentrations obtained for the six volunteers ranged from 209-455 ng/ml, and were reached 0.75-2.5 hours following the naltrexone dose. The elimination half-life values for  $6\beta$ -naltrexol ranged from 14.5 to 37.4 hours, with a mean value of  $20.8 \pm 8.4$  hours. The coefficient ( $r^2$ ) for the determination of elimination equation was greater than 0.98 for  $6\beta$ -naltrexol, while for naltrexone the  $r^2$  was greater than 0.97 for all volunteers.

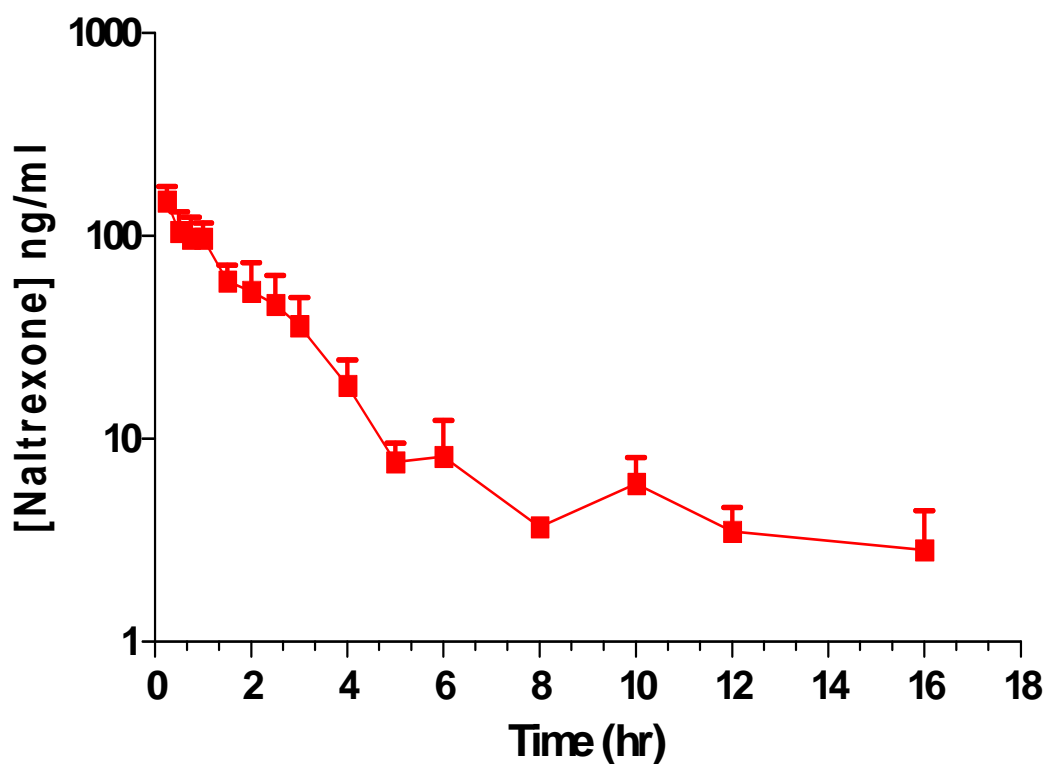


Figure 5.5 Mean ( $\pm$ SEM) naltrexone concentration in saliva vs time profiles (up to 16 hours) for six volunteers following an oral dose of naltrexone (50 mg). Note semi-log scale.

Table 5.12 Individual data of salivary pH values, saliva naltrexone and 6 $\beta$ -naltrexol concentrations following an oral dose of naltrexone (50 mg) in volunteers #1, #2, and #3.

Time (hours)	Volunteer #1			Volunteer #2			Volunteer #3		
	pH	Ntx*	6 $\beta$ -nol**	pH	Ntx*	6 $\beta$ -nol**	pH	Ntx*	6 $\beta$ -nol**
Baseline	6.47	-	-	6.8	16	-	6.52	-	-
0.25	6.54	83	-	6.81	138	-	6.4	249	11
0.5	6.38	155	60	6.97	79	37	6.62	216	162
0.75	6.64	71	67	6.85	70	148	6.76	148	455
1.0	6.43	159	178	6.96	52	266	6.59	124	297
1.5	4.75	98	181	6.81	36	368	6.6	69	306
2.0	6.08	149	209	6.8	25	327	6.35	23	189
2.5	6.43	116	160	6.87	17	163	6.63	22	189
3.0	6.62	88	123	6.78	11	169	6.15	9	83
4	6.41	46	72	6.91	8	112	6.53	12	127
5	5.68	12	101	6.67	-	38	6.39	7	82
6	6.32	27	199	6.6	-	100	6.19	4	51
8	5.21	4	61	6.3	3	77	6.14	3	51
10	6.16	14	107	6.58	10	45	6.02	4	45
12	5.85	8	57	6.32	4	48	6.11	2	32
16	6.44	10	67	6.18	-	48	6.52	3	43
24	6.35	3	51	6.59	10	63	6.49	-	30
30	6.32	-	29	6.68	6	43	6.11	-	22
48	6.11	-	22	6.57	8	25	6.41	-	-
60	5.94	-	22	6.62	4	13	6.17	-	-

\*= saliva naltrexone (ng/ml)

\*\*= saliva 6 $\beta$ -naltrexol (ng/ml)

- = <LOQ

Table 5.13 Individual data of the salivary pH values, saliva naltrexone and 6 $\beta$ -naltrexol concentrations following an oral dose of naltrexone (50 mg) in volunteers #4, #5 and #6.

Time (hrs)	Volunteer #4			Volunteer #5			Volunteer #6		
	pH	Ntx*	6 $\beta$ -nol**	pH	Ntx*	6 $\beta$ -nol**	pH	Ntx*	6 $\beta$ -nol**
Baseline	6.71	-	-	6.79	23	-	7.18	-	-
0.25	6.79	178	-	6.40	166	-	7.01	74	-
0.5	6.55	57	25	6.68	68	-	6.81	54	27
0.75	6.41	203	102	6.44	49	48	6.54	44	87
1.0	6.74	112	150	6.45	100	217	6.85	37	202
1.5	6.56	38	98	6.4	87	445	6.88	30	243
2.0	6.70	61	153	6.50	48	374	6.54	13	161
2.5	6.67	85	218	5.60	24	204	6.69	11	144
3.0	6.73	66	218	6.06	30	289	6.64	12	181
4	6.66	23	131	6.37	17	201	6.89	4	58
5	6.63	13	102	6.00	7	92	6.34	7	84
6	6.43	11	82	6.42	7	109	6.67	-	73
8	6.78	4	49	5.91	5	83	6.59	3	52
10	6.52	5	36	6.29	3	81	6.52	-	48
12	6.66	3	42	6.15	4	56	6.58	-	38
16	6.72	4	47	6.38	-	32	6.55	-	30
24	6.70	-	27	6.51	-	41	6.73	9	26
30	6.53	-	22	6.54	-	31	6.63	-	23
48	6.59	-	13	6.46	-	15	5.67	-	-
60	6.56	-	-	6.39	-	-	6.27	-	-

\*= saliva naltrexone (ng/ml)

\*\*= saliva 6 $\beta$ -naltrexol (ng/ml)

- = <LOQ



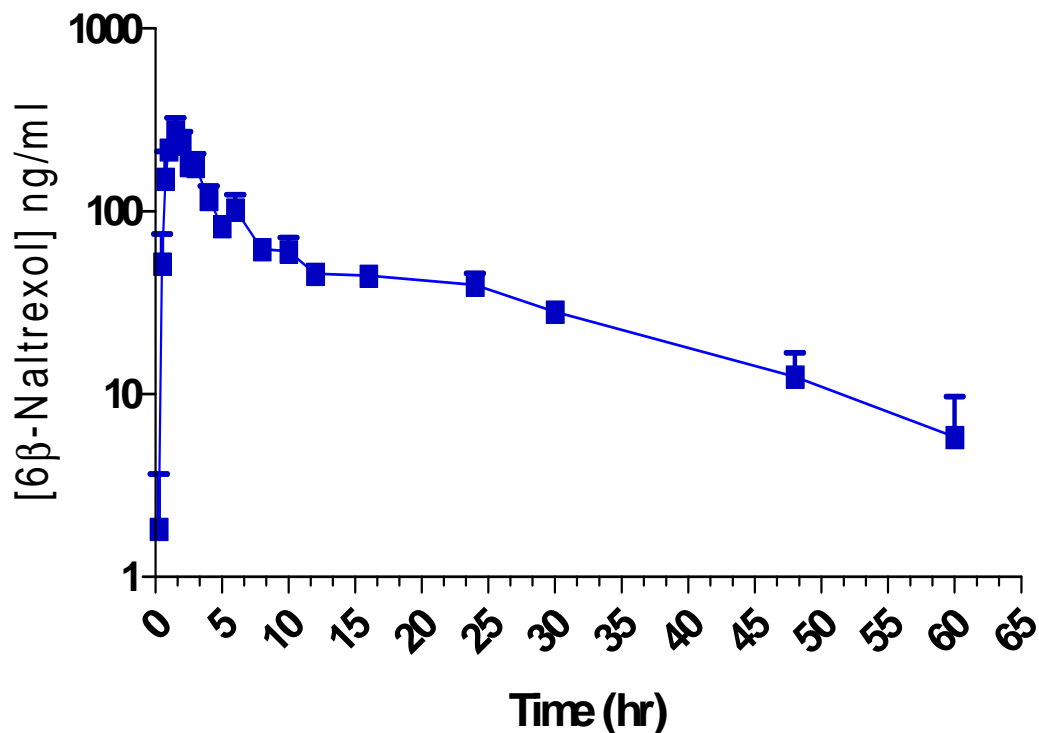


Figure 5.6 Mean ( $\pm$  SEM) 6 $\beta$ -naltrexol concentration in saliva vs time profiles for six volunteers following an oral dose of naltrexone (50 mg). Note semi-log scale

#### 5.4.4.4. Saliva:Plasma concentration ratio

Figure 5.7 shows the mean saliva:plasma concentration ratios of naltrexone and 6 $\beta$ -naltrexol in the six volunteers following an oral dose of 50 mg of naltrexone. The latter were lower than the corresponding naltrexone ratios, due to the higher plasma 6 $\beta$ -naltrexol concentrations even though the saliva 6 $\beta$ -naltrexol concentrations were higher than those obtained for naltrexone. Figure 5.8 shows the individual profiles for naltrexone measured in saliva and plasma for the six volunteers. The pH of the saliva samples had no effect on saliva/plasma ratios, with the regression coefficient ( $r^2$ ) being 0.32 for naltrexone and 0.14 for 6 $\beta$ -naltrexol.

Table 5.16 Mean ( $\pm$ SD) pharmacokinetic values for naltrexone and 6 $\beta$ -naltrexol in saliva following an oral dose of naltrexone (50 mg).

	T <sub>max</sub> (hr)	C <sub>max</sub> ng/ml	Half-life (hr)	AUC	$\frac{AUC_{saliva}}{AUC_{plasma}}$
Naltrexone	0.46 (0.33)	164 (59)	2.9 (0.3)	363 (141)	19.5 (8.3)
6 $\beta$ -naltrexol	1.63 (0.59)	323 (114)	20.7 (8.4)	2349 (680)	3.5 (1.2)

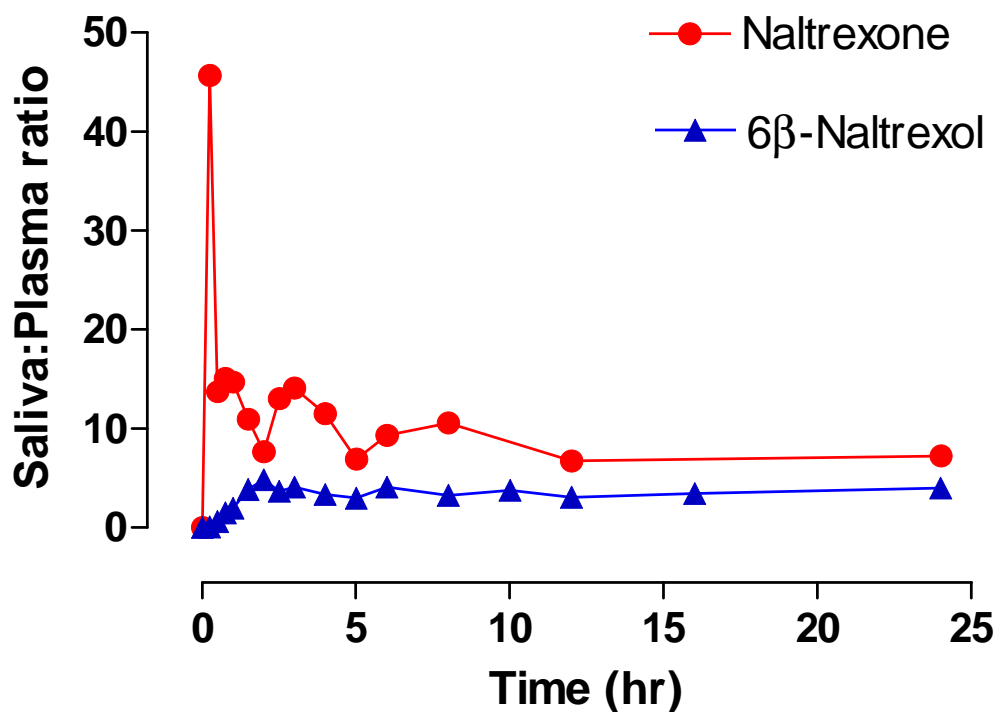


Figure 5.7 Mean saliva/plasma concentration ratios of naltrexone (red circles) and 6 $\beta$ -naltrexol (blue triangles) in six healthy volunteers after 50 mg oral naltrexone.

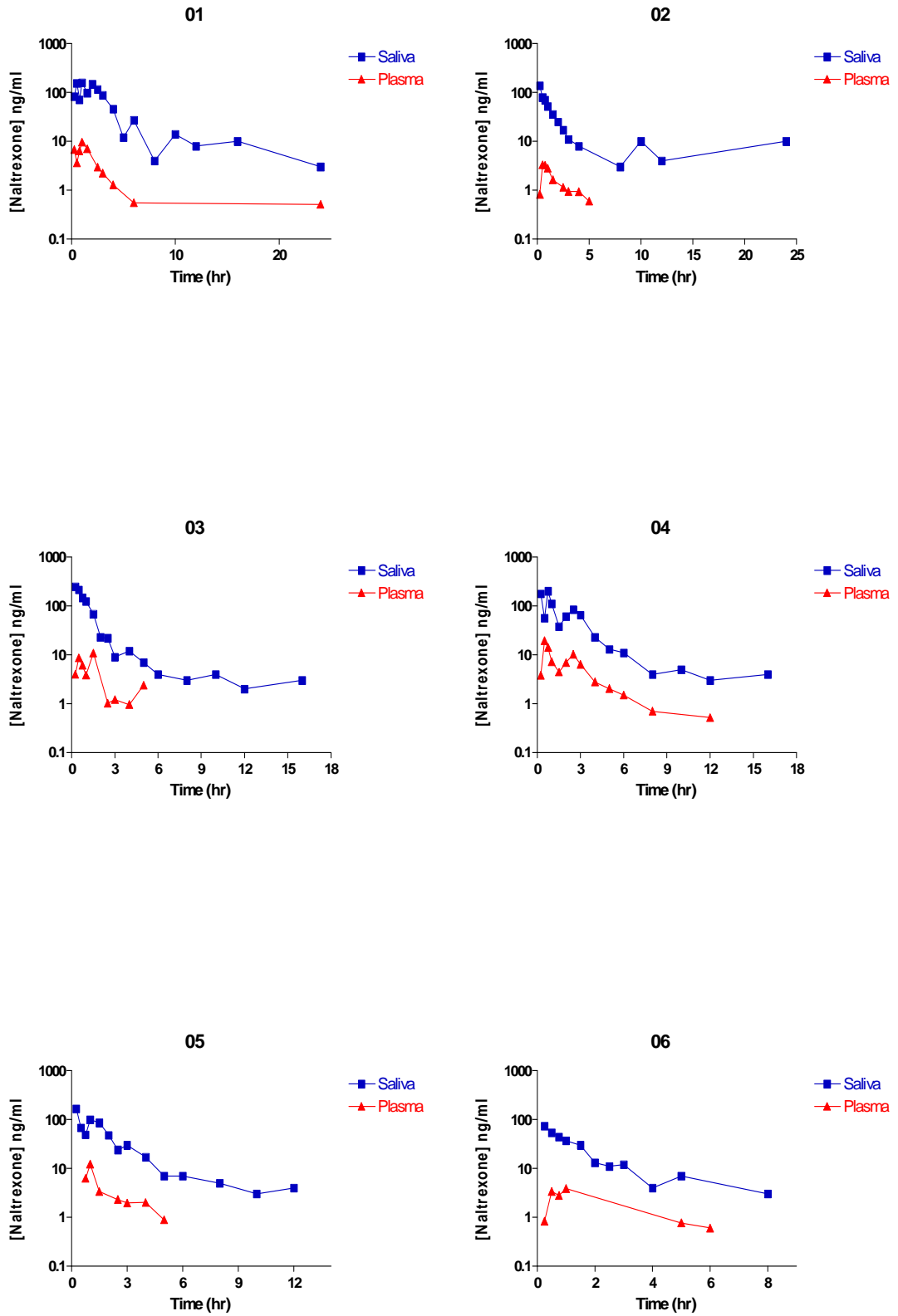


Figure 5.8. The saliva and plasma naltrexone profiles for the six volunteers following an oral dose of naltrexone (50 mg).

#### 5.4.5. Plasma protein binding

The plasma protein binding of naltrexone and 6 $\beta$ -naltrexol was determined following the oral dose of naltrexone in all volunteers. For naltrexone, the binding ranged from 9 to 18% (mean 15.1  $\pm$  3.6%), over the concentration range of 4-20 ng/ml. 6 $\beta$ -Naltrexol was more highly bound, ranging from 23 to 31% (mean 26.1  $\pm$  3.1%), at concentrations ranging from 4-160 ng/ml. The results were not corrected for non-specific binding as in the control study at 10 and 100 ng/ml naltrexone and 6 $\beta$ -naltrexol non-specific binding was insignificant. The non-specific protein binding at 100 mg/ml of analyte were approx 2 and 5% for naltrexone and 6 $\beta$ -naltrexol, respectively.

#### 5.4.6. Blood:Plasma Concentration Ratio

The blood:plasma concentration ratios were calculated for the 30-minute sample taken after both oral (50 mg) and intravenous (1 mg) naltrexone. There were no significant differences between the ratios after oral or IV treatment for either naltrexone or 6 $\beta$ -naltrexol. The mean ( $\pm$ SD) ratios for naltrexone were 2.79  $\pm$  0.25 and 1.87  $\pm$  0.24, respectively, for oral and IV treatment, and for 6 $\beta$ -naltrexol, 2.04  $\pm$  0.14 and 3.47  $\pm$  0.72, respectively, after oral and IV treatment.

#### 5.4.7. Side-Effects and Mood Evaluation

##### 5.4.7.1. Profile of Mood States

There were no significant effects of drug (oral vs IV), or time for the measurements of tension/anxiety, depression/dejection ( $p=0.076$ ), anger/hostility ( $p=0.231$ ), vigour/activity, fatigue/inertia or total mood changes ( $p=0.47$ ) compared to baseline for the subjects. The measurements of confusion/bewilderment showed no effect over time, but the IV dose resulted in significantly increased confusion/bewilderment than the oral naltrexone dose. This was evident in the increase in scores obtained in the first (baseline) questionnaire, and may have been due to anxiety of receiving an intravenous medication. Additionally, there was no increase in any of the measured parameters with increased concentrations of naltrexone or 6 $\beta$ -naltrexol in the plasma, urine or saliva.

##### 5.4.7.2. Methadone Symptoms Checklist

There were no significant drug and time effects for the parameters tested with the methadone symptoms checklist. One volunteer fainted after receiving the intravenous dose of naltrexone,

and was treated accordingly by the medical officer present. At one hour post-dose this volunteer felt dizzy, but this effect was not present at three hours post-dose. A different volunteer complained of having a dry mouth with both oral and IV doses of naltrexone which was not considered a drug effect as it was present at the baseline sample. No other symptoms could be attributed to the administration of naltrexone either orally or intravenously. As with the POMS data, increased concentrations of naltrexone and 6 $\beta$ -naltrexol in plasma, urine or saliva had no effect on the parameters tested in the questionnaire. The most often reported symptom was tiredness, which was noted late in the evening on both study occasions, and early in the mornings on several occasions. As these volunteers were all opioid naïve, these tests were performed to show that naltrexone did not alter mood.

### **5.5. Discussion**

The assays used for the simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in plasma, saliva and urine in the current study continued to perform with high degrees of accuracy and precision, as did the assays for the measurement of high concentrations of analytes in urine.

In the current study, the plasma concentrations of naltrexone after an intravenous dose of 1 mg were quantifiable only up to 4 hours post-dose in all but one of the volunteers who had quantifiable levels to 12 hours. These findings are similar to those obtained by Wall *et al*, (1981) of detectable concentrations to 5-6 hours following the same dose. Plasma concentrations of 6 $\beta$ -naltrexol were unquantifiable following IV naltrexone in the current study, whereas Wall *et al* (1981) reported plasma 6 $\beta$ -naltrexol concentrations both conjugated and unconjugated, but these concentrations were all below 1 ng/ml, which would have been deemed unquantifiable with the assay used in the current study, where the limit of quantification for 6 $\beta$ -naltrexol was 2 ng/ml. In the current study plasma samples were not subjected to hydrolysis, although Wall *et al* (1981) reported higher concentrations of unconjugated than conjugated 6 $\beta$ -naltrexol. In retrospect, a dose of naltrexone in the order of 5 or 10 mg would have resulted in quantifiable concentrations with this assay system. However, the 1 mg IV dose had been approved by the Ethics Committee (it is unknown whether the Committee would have accepted the higher doses). In agreement with the study by Wall *et al* (1981), the major urinary metabolites observed after the IV dose were unconjugated 6 $\beta$ -naltrexol and conjugated naltrexone and 6 $\beta$ -naltrexol. The total per cent of the dose excreted as naltrexone and 6 $\beta$ -naltrexol in 96 hours in the current study after one mg of IV naltrexone was 55%, which is comparable to the value obtained by Wall *et al* (1981) of 52% for these two compounds to 72 hours.

It is unknown why one volunteer appeared to excrete more than double the one mg IV dose administered. Although his plasma AUC was higher than that of the other volunteers, the  $C_{\max}$  value was the same as that of the other volunteers suggesting the amount of naltrexone he received was not different. As is evident from data in tables 5.7 and 5.8, even excluding volunteer #6, there is large interindividual variation in the excretion profiles.

The mean total plasma clearance of naltrexone for five of the six volunteers after iv administration in this study was 4.7 ( $\pm 5.3$ ) L/min, and 6.0 ( $\pm 4.4$ ) L/min with respect to blood clearance. This exceptionally large value exceeds liver blood flow (1.5 L/min). This value may be an overestimation resulting from an underestimation of the AUC due to the inability to quantify naltrexone after 12 hours post-dose, and thus missing the late terminal phase. Renal excretion accounts for a minor proportion of clearance, indicating large extrahepatic metabolism of naltrexone in humans.

In the current study, following a 50 mg oral dose of naltrexone, the maximum concentrations of naltrexone and 6 $\beta$ -naltrexol were obtained 0.9 and 0.8 hours, respectively, after the naltrexone dose. These  $T_{\max}$  values are similar to those obtained by Meyer *et al*, (1984) of 0.95 and 0.91 hours for naltrexone and 6 $\beta$ -naltrexol, respectively. Verebey *et al* (1976) obtained slightly longer  $T_{\max}$  values of 1 and 2 hours for naltrexone and 6 $\beta$ -naltrexol respectively, after 100 mg of oral naltrexone, whereas Wall *et al* (1981) reported much longer  $T_{\max}$  values of 2.5 and 6.5 hours for naltrexone and 6 $\beta$ -naltrexol, respectively following an oral dose of 50 mg of naltrexone. However, the plasma concentration profiles in the latter study appear to be much flatter than in any of the other studies, perhaps due to the use of radioactive methodology.

The mean  $C_{\max}$  of naltrexone after an oral dose of 50 mg was  $8.0 \pm 6.7$  ng/ml in the current study, which is very similar to that obtained by Meyer *et al* (1984) of  $8.55 \pm 4.84$  ng/ml, but much lower than Wall *et al* (1981) value of 73.9. There was large variation in results for all studies. In contrast, the maximum 6 $\beta$ -naltrexol concentrations obtained in the current and Meyer studies ( $127 \pm 36$  ng/ml in the current study and  $99.3 \pm 30.2$  ng/ml by Meyer *et al*), were moderately higher than those obtained by Wall *et al* (1981) of 76 ng/ml. Interestingly, Verebey *et al* (1976) obtained  $C_{\max}$  values of  $43.6 \pm 29.9$  and  $87.2 \pm 25$  ng/ml for naltrexone and 6 $\beta$ -naltrexol after an oral 100 mg dose of naltrexone. One explanation for the difference in proportion of metabolite formed could be that at higher doses there is less conversion of the

parent drug to the metabolite, suggesting perhaps that the metabolic reduction of naltrexone to 6 $\beta$ -naltrexol is a saturable process. In the initial studies of cytosolic formation of 6 $\beta$ -naltrexol from naltrexone reported in Chapter 4 of this thesis, 250  $\mu$ M of substrate (naltrexone) was used, which is equivalent to 137 mg. At this concentration of substrate, there was a loss of linearity of the velocity/substrate vs velocity Eadie Hofstee plots, this was not seen when the maximum substrate concentration was 150  $\mu$ M (equivalent to approximately 51 mg). While factors other than concentration alone need to be taken into account when extrapolating from the *in vitro* data, it is possible that the enzymatic formation of the 6 $\beta$ -naltrexol metabolite is a saturable process at doses given clinically.

In the current study, as shown in Fig 5.4, the plasma concentrations of 6 $\beta$ -naltrexol were higher than those of naltrexone at all time points following the oral dose in agreement with Meyer *et al* (1984), and the mean AUC of 6 $\beta$ -naltrexol was 35 times higher than that of naltrexone.

Following administration of an oral dose of naltrexone of 50 mg, the major urinary excretion products were conjugated and unconjugated 6 $\beta$ -naltrexol, followed by conjugated naltrexone. The naltrexone was excreted mostly as the glucuronide conjugate whereas the 6 $\beta$ -naltrexol was excreted mostly unconjugated. This pattern was also seen after IV naltrexone administration. These results agree with those obtained by Cone *et al* (1974) and Wall *et al*, (1981) who also administered 50 mg of naltrexone orally. The excretion of both products accounted for only 35% of the dose, which is comparable to that obtained previously by others, except Verebey *et al* (1976), who recovered over 70% of a daily dose of 100 mg of naltrexone as naltrexone and metabolites, including 2-hydroxy-3-methoxy-naltrexol in urine and faeces (Verebey *et al.*, 1976). The rest of the dose could be in unmeasured intermediate metabolites, or N-dealkylated metabolites. (Verebey *et al.*, 1976).

As previously stated, the renal clearance of naltrexone has been reported to be similar to that of inulin, suggesting the mechanism is by filtration only. The renal clearance ratio to inulin of 2 obtained for 6 $\beta$ -naltrexol indicates clearance is by tubular secretion (Wall, 1981). In a study by Verebey *et al*, (1976), the mean ( $\pm$ SD) renal clearance value of 67 ( $\pm$ 38) ml/min for naltrexone following oral administration of 100 mg, indicate that naltrexone is partially reabsorbed. The corresponding values for 6 $\beta$ -naltrexol, were 318 ( $\pm$ 70) ml/min following 100 mg of naltrexone orally. The authors concluded that 6 $\beta$ -naltrexol is thus actively secreted by

kidney tubules. Similar renal clearance results for naltrexone ( $78 \pm 37$  ml/min) and  $6\beta$ -naltrexol ( $420 \pm 108$  ml/min) were obtained in the current study after an oral dose of 50 mg, in agreement with Verebey's conclusion of filtration of naltrexone and active tubular secretion of  $6\beta$ -naltrexol.

The degree of plasma protein binding for naltrexone obtained in the present study of 15% is not dissimilar to that previously reported by Ludden *et al* (1976) of 20%. The plasma binding of  $6\beta$ -naltrexol following an oral dose of naltrexone in the current study is significantly higher than that of naltrexone at 26%. However, these low degrees of plasma binding are of no major pharmacokinetic consideration and are similar to other phenanthrene opioids such as morphine 35% and oxycodone (45%) (Leow *et al.*, 1993).

Both naltrexone and  $6\beta$ -naltrexol were unquantifiable in saliva after an IV naltrexone dose of one mg. However, following the 50 mg oral dose, salivary concentrations were higher than those measured in the plasma, with the AUC saliva:AUC plasma of 19.5 and 3.5 for naltrexone and  $6\beta$ -naltrexol, respectively. This finding was also reported previously by Verebey *et al* (1980) in two subjects receiving 400 mg naltrexone daily. Additionally, the mean ( $\pm$  SD) half-life of naltrexone in saliva ( $2.92 \pm 0.31$ ) was significantly longer ( $p=0.013$ ) than that calculated for naltrexone in plasma ( $1.88 \pm 0.47$ ). In one subject, the naltrexone concentrations in saliva following the oral 50 mg dose were higher than for all other subjects. This subject possibly had some naltrexone in his mouth following the dose, even though the subjects did rinse out their mouths following the oral dose. The half-life of  $6\beta$ -naltrexol in saliva was also higher than that in plasma, but was not statistically significant. The HPLC method reported in the current study requires much less sample manipulation than the GC/MS method reported previously for the quantification of naltrexone and  $6\beta$ -naltrexol in saliva (Verebey, 1980). The saliva profiles mirrored those of the plasma for both naltrexone and  $6\beta$ -naltrexol, and being less invasive than venepuncture, could be an easier alternative for monitoring naltrexone status.

The blood:plasma ratios for naltrexone and  $6\beta$ -naltrexol after both oral and iv treatment were all greater than 1, indicating greater distribution into the blood cells. In a previous study where whole blood samples taken 16 and 24 hours after administration from four subjects receiving 2x200 mg of naltrexone were analysed, the blood:plasma ratios for naltrexone were less than 1 in all samples. In this study,  $6\beta$ -naltrexol was not recovered in red blood cell



extracts using chloroform extractions of PFPA derivatised samples (Verebey, 1980). The differences in results could be due to the very different assay methods, or the large difference in the doses administered between the two studies.

Faeces samples were not collected during this study as Verebey *et al* (1976) showed <5% of the dose was accounted for by this route and Wall *et al* (1981) reported 5.4% of excretion in the faeces following an oral dose of naltrexone of 50 mg. Additionally, the 2-hydroxy-3-methoxy metabolites of naltrexone and 6 $\beta$ -naltrexol were not quantified in any of the samples, due to a lack of the pure compounds. There were additional peaks observed on the chromatograms of the urine and plasma samples from the oral dose which are likely to be 2-hydroxy-3-methoxy-naltrexol, which while larger than the naltrexone peaks were significantly smaller than those of 6 $\beta$ -naltrexol.

Whereas the long duration of action of naltrexone has been regarded as being due, in part, to the human metabolite 6 $\beta$ -naltrexol (Cone *et al.*, 1974; Verebey *et al.*, 1976), it is not known whether the lack of positive outcomes in some patients (O'Malley *et al.*, 1996), and patient compliance could be related to plasma 6 $\beta$ -naltrexol concentrations. Hence, knowledge of the factors causing variability in the formation of 6 $\beta$ -naltrexol could be of importance for individualised therapy optimisation.

The findings of King *et al* (1997) that those volunteers who reported one or more subjective side effects had significantly higher urinary concentrations of 6 $\beta$ -naltrexol, was not reproduced in this study for urine, plasma or saliva. The sample size of the previous study however, was larger than that of the current study (24 versus 6).

In conclusion, the results reported in this chapter show that there is large variation in the pharmacokinetic parameters tested between individuals and that clearance values indicate considerable extrahepatic mechanisms. Saliva is possibly a better alternative to plasma in assessing naltrexone status following the 50 mg daily dose used clinically.

## 6. *In vivo* and *in vitro* potency studies of 6 $\beta$ -naltrexol.

### 6.1. Introduction

As previously stated, in humans, quantitatively the major metabolite of naltrexone is 6 $\beta$ -naltrexol (Chatterjee et al., 1974, and Chapter 5, this thesis). Plasma 6 $\beta$ -naltrexol concentrations after an oral naltrexone dose are considerably higher than those of naltrexone as shown in Chapter 5 (Bertolotti et al., 1997; Meyer et al., 1984; Wall, 1981) in all subjects except those with compensated or decompensated cirrhosis (Bertolotti et al., 1997). In addition, 6 $\beta$ -naltrexol appears to remain much longer in the systemic circulation than naltrexone (Wall et al., 1984).

It has been suggested that as an active metabolite, 6 $\beta$ -naltrexol could account for the long duration of action of naltrexone in humans (Cone et al., 1974; Verebey et al., 1976). As has been shown with benzodiazepines, when an active metabolite has a much longer half-life than the parent drug, the metabolite will accumulate more than the parent drug, and is likely to be responsible for, or at least contribute to either the therapeutic or adverse events (Garattini, 1985).

In addition to its potential therapeutic effect, 6 $\beta$ -naltrexol may also contribute to the side-effects associated with naltrexone administration. In a study in moderate to heavy social drinkers, urinary concentrations of 6 $\beta$ -naltrexol three hours after an acute 50 mg oral dose of naltrexone, were significantly higher in subjects who reported subjective side-effects compared to those who reported no side-effects, whereas no differences were found between the groups in urinary naltrexone concentrations (King et al., 1997).

In determining the role of 6 $\beta$ -naltrexol in the therapeutic and adverse effects of naltrexone, it is necessary to determine both the relative potency of the metabolite and its duration of action. Potency studies of naltrexone were originally conducted in comparison to naloxone which was already in clinical use. In the guinea pig ileum preparation, naltrexone was shown to be 3.5 to 5 times more potent than naloxone (Takemori and Portoghese, 1984), but there are no data on the relative potencies of naltrexone and 6 $\beta$ -naltrexone using *in vitro* preparations. While important information can be obtained from such *in vitro* studies, the results cannot necessarily be extrapolated to the whole organism; *in vivo* studies are therefore important. However, when interpreting the results of *in vivo* studies, it needs to be recognised that the metabolism of naltrexone shows marked species variation. There have been some 6 $\beta$ -naltrexol potency studies conducted previously in animal models with varying results. *In vivo*, 6 $\beta$ -naltrexol was only 1/85<sup>th</sup> as potent as naltrexone in achieving the same degree of antagonism of oxymorphone-

induced loss of righting reflex in rats (Chatterjie et al., 1975). In the chronic spinal dog preparation, 6β-naltrexol was only 1/12<sup>th</sup> to 1/15<sup>th</sup> as potent as naltrexone in precipitating the abstinence syndrome (Cone et al., 1974). In a study measuring the number of jumps produced after administration of antagonist to morphine-dependent mice, 6β-naltrexol was 1/53<sup>rd</sup> as potent as naltrexone (Fujimoto et al., 1975). 6β-Naltrexol was four times longer-acting than naltrexone in preventing the loss of righting reflex in morphine-dependent rats (Blumberg and Ikeda, 1978), and nine times longer-acting in blocking the morphine-induced Straub tail reaction in mice (Blumberg and Ikeda, 1978).

As previously stated, there are no comparative *in vitro* studies between naltrexone, 6β-naltrexol and naloxone, the prototype mu antagonist, and there is substantial variability in potency in these previously reported *in vivo* studies.

## **6.2. Aim**

The aim of the current study was to assess the potency of 6β-naltrexol compared to naltrexone and naloxone, a) in blocking morphine-induced reduction of twitch height in electrically-stimulated guinea pig ileum; and b) using the hotplate test to assess *in vivo* antagonist activity in reversing morphine-induced antinociception in mice. In addition, the duration of antagonist activity was determined *in vivo* for each of the three compounds.

## **6.3. Materials and Methods**

### **6.3.1. Chemicals**

Naltrexone HCl, naloxone HCl and morphine HCl were purchased from Sigma Aldrich (St Louis, MO, USA). 6β-Naltrexol was synthesised as the free base in the Department of Chemistry, University of Adelaide by the method of Chatterjie and co-workers (1975) (see Chapter 2). Sodium chloride, potassium orthophosphate, D-glucose and calcium chloride were purchased from Merck Pty Ltd, (Kilsythe, Australia). Potassium chloride, sodium hydrogen carbonate and magnesium chloride were purchased from Ajax Chemicals (Auburn, Australia). The Krebs solution used contained NaCl 118.1mM, KCl 4.7mM, KH<sub>2</sub>PO<sub>4</sub> 1.18mM, NaHCO<sub>3</sub> 25mM, D-glucose 5.55mM, CaCl<sub>2</sub> 2.52mM and MgCl<sub>2</sub> 1.05mM.

Ethics approval for the following studies was obtained from the University of Adelaide Animal Ethics Committee (approval number M/038/97).

### **6.3.2. *In vitro* potency study**

Sections (2 cm) of intact ileum from adult male guinea pigs were suspended via stainless steel electrodes at a resting tension of 1g in a carbogenated Krebs bath maintained at 32°C. The electrodes were connected to a transducer (Harvard, App. Co. Inc. Mills, Mass., USA, modified for MacLab) which delivered 50 volts at 0.1 pulses/sec. The entire apparatus was connected to a computer to record twitch height, and the data collected by the Maclab program (ADInstruments, Castle Hill, Australia).

The antagonists naltrexone HCl, naloxone HCl and 6 $\beta$ -naltrexol were added as aqueous solutions to achieve concentrations of 10<sup>-11</sup> to 10<sup>-4</sup> M, 15 minutes prior to determination of cumulative concentration-response curves with morphine (10<sup>-11</sup> to 10<sup>-4</sup> M).

### **6.3.3. *In vivo* potency study**

#### **6.3.3.1. Animals**

All experiments were conducted on adult, male Swiss Albino mice with a mean weight of 40 grams. The mice were purchased from the Animal Resource Centre (Adelaide, Australia), and were housed in groups of 4-8 in a temperature-controlled environment maintained at 22°C, with a 12/12 hour light/dark cycle. Food (standard laboratory mouse chow) and water were available *ad libitum*. All hotplate experiments were conducted between 0900 and 1200, with at least one week between experiments to minimise any learning effect. Ten mice were used for each antagonist at each dose in a randomised design.

#### **6.3.3.2. Preparation of solutions for injection**

Drugs were dissolved in 0.9% sodium chloride to achieve stock solutions of 10 mg/ml of the free-base. They were stored at 4 °C, warmed to room temperature before dilution and administered intraperitoneally (ip) in a volume of 10 ml/kg.

#### **6.3.3.3. Hotplate Response**

The mice were placed on an electrically-controlled temperature hotplate (University of Adelaide, Australia) measuring 30 cm by 30 cm, surrounded by removable plexiglass sides. The hotplate temperature was maintained at 50 ± 0.2 °C, and mice remained on the plate for a maximum of 60 seconds. To avoid excess movement on the hotplate, the mice were confined under a 500 ml glass beaker placed on the hotplate. Responses were measured as the latency (seconds) for the mice to lick, raise or shake their paws.

#### 6.3.3.4. Acute Potency Studies

Mice were weighed and then placed on the hotplate to obtain a baseline response. One mouse was administered morphine alone (30 mg/kg, approximate ED<sub>80</sub> from preliminary experiments) on each study session to serve as a control. If the baseline value for this mouse was at least 45 sec, then all other mice were administered an antagonist (naloxone, naltrexone or 6 $\beta$ -naltrexol) in random order at 7-9 doses ranging from 0.001 to 30 mg/kg. Morphine was administered at a dose of 30 mg/kg (approximate ED<sub>80</sub> from preliminary experiments) 15 minutes after the antagonist. The hotplate responses (seconds) were recorded 30 and 45 minutes post-antagonist (15 and 30 minutes post-morphine).

#### 6.3.3.5. Duration of Effect Study

Mice were weighed and then placed on the hotplate to obtain a baseline response. One mouse was administered morphine alone (30 mg/kg) during each study session to obtain a control response time. An initial study was conducted whereby the mice were administered the highest dose of antagonists (30 mg/kg) from the acute study. The hotplate tests were conducted 15, 45, 90, 180 and 270 minutes post-morphine. The experiment was repeated using the ID<sub>50</sub> (the dose of antagonist that resulted in a 50% decrease in hotplate retention time) values obtained 30 minutes post-antagonist in the acute potency studies (naltrexone 0.007 mg/kg, naloxone 0.016 mg/kg and 6 $\beta$ -naltrexol 1.30 mg/kg). The mice were pre-administered the antagonist 45, 90, 120, 180 and 1080 minutes prior to morphine (30 mg/kg). They were then tested on the hotplate 15 minutes post-morphine administration. Ten mice were tested at each time point for each antagonist.

#### 6.3.4. Data Analysis

For the *in vitro* study, morphine EC<sub>50</sub> values (concentration at which the electrically-stimulated twitch height is reduced by 50%) were determined. IC<sub>50</sub> (concentration of antagonist which produced a 50% reduction in morphine-induced inhibition of twitch height) and K<sub>i</sub> (the concentration of antagonist required to increase the EC<sub>50</sub> of morphine two-fold) values were determined using non-linear least squares regression analyses of the log of the antagonist concentration vs the agonist concentration. Schild plots (pA<sub>2</sub>), were constructed of the log of the antagonist concentration against log of the dose-ratio -1 (the dose ratio is IC<sub>50</sub> with antagonist/IC<sub>50</sub> without antagonist), using GraphPad Prism software (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, USA).

For the *in vivo* study, all hotplate times were corrected for their baseline values (control

latencies). The responses were expressed as a per cent of the maximum time the animals remained on the hotplate compared to the time on the hotplate after morphine alone (maximum possible response, MPR), calculated as:

$$\% \text{ Inhibition} = \frac{(\text{test latency} - \text{control latency})}{(\text{MPR} - \text{control latency})} \times 100$$

ID<sub>50</sub> values were calculated using the equation:

$$\% \text{ Inhibition} = \frac{I_{\max} * \text{Dose}^n}{ID_{50}^n + \text{Dose}^n}$$

Where I<sub>max</sub> is the maximum obtained response, n is the slope factor and ID<sub>50</sub> represents the response at 50% of maximum. All data are expressed as mean  $\pm$  SEM response time. For the duration of effect study, the responses obtained for antagonist administration 45 min pre-morphine administration were corrected to 1, and the subsequent time points were calculated relative to that value. The time for the inhibition to decrease by 50% (duration of action) was calculated as the time taken for the relative antagonism to decrease by half from the first time point at 45 min post-antagonist dose.

## 6.4. Results

### 6.4.1. *In vitro* potency studies

Figure 6.1 shows an example of the concentration-response curves obtained for 6 $\beta$ -naltrexol antagonism of morphine-induced twitch height in the guinea pig ileum, with representative Schild plots for each of the antagonists in Figure 6.2. The mean EC<sub>50</sub> for morphine (n=20) obtained in these experiments was 1.63x10<sup>-8</sup> M. Table 6.1 shows the antagonist K<sub>i</sub> values obtained using the Schild plots, the means of which were 416 $\pm$ 149 (n=6) pM, 265 $\pm$ 101 (n=8) pM and 94 $\pm$ 25 (n=6) pM, for naloxone, naltrexone and 6 $\beta$ -naltrexol, respectively. Assigning naloxone a potency of 1 resulted in relative mean potency values of 4.5 and 1.7 for 6 $\beta$ -naltrexol and naltrexone, respectively.

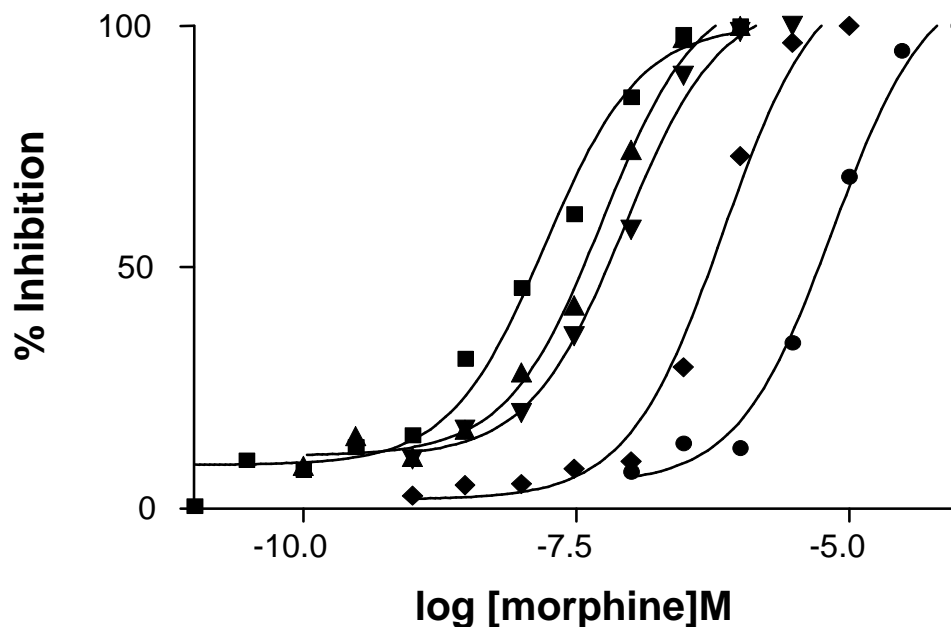


Figure 6.1 The mean ( $n=6$ ) per cent inhibition by 6 $\beta$ -naltrexol of the morphine-induced reduction of twitch height in electrically stimulated guinea pig ileum. (6 $\beta$ -naltrexol concentrations,  $\blacksquare$ -control,  $\blacktriangle$   $10^{-10}$  M,  $\blacktriangledown$   $10^{-9}$  M,  $\blacklozenge$   $10^{-8}$  M,  $\bullet$   $10^{-7}$  M).

Table 6.1 Antagonist  $K_i$  values (pM) for naloxone, naltrexone and 6 $\beta$ -naltrexol in antagonising morphine-induced reduction of twitch height in guinea pig ileum.  $N=6$  for naloxone and 6 $\beta$ -naltrexol,  $n=8$  for naltrexone.

	Naloxone ( $\times 10^{-12}$ )	Naltrexone ( $\times 10^{-12}$ )	6 $\beta$ -naltrexol ( $\times 10^{-12}$ )
	93.0	315	43
	339	52	111
	21.4	136	200
	1030	27	102
	578	581	26
	432	812	83
		87	
		109	
<b>Mean</b>	<b>416</b>	<b>265</b>	<b>94</b>
<b>SEM</b>	<b>149</b>	<b>101</b>	<b>25</b>

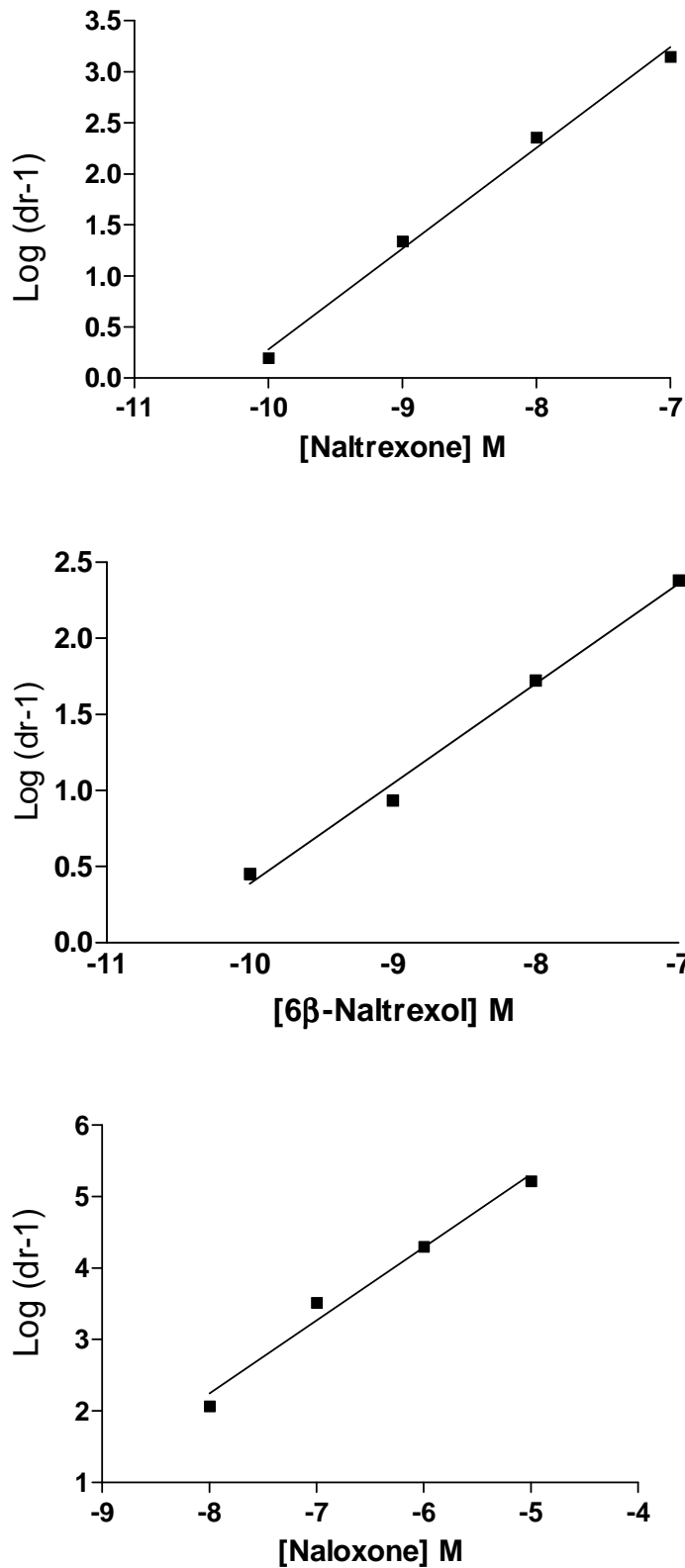


Figure 6.2. Representative Schild plots for naltrexone,  $6\beta$ -naltrexol and naloxone in inhibiting morphine-induced twitch height in electrically stimulated guinea pig ileum.



**6.4.2. *In vivo* potency studies****6.4.2.1. Acute Potency Study**

Figure 6.3 summarises the dose-related inhibition of morphine-induced antinociception by naltrexone, naloxone and 6 $\beta$ -naltrexol. Hotplate latency was measured 15 and 30 minutes post-morphine, and 30 and 45 minutes post antagonist administration. Only the 30 minute post-antagonist data are presented as there were no significant differences in response between the two time points. The mean ID<sub>50</sub> values obtained were: naltrexone 0.007 mg/kg, naloxone 0.016 mg/kg, and 6 $\beta$ -naltrexol 1.30 mg/kg. Naltrexone was on average twice as potent as naloxone, and 185 times as potent as 6 $\beta$ -naltrexol.

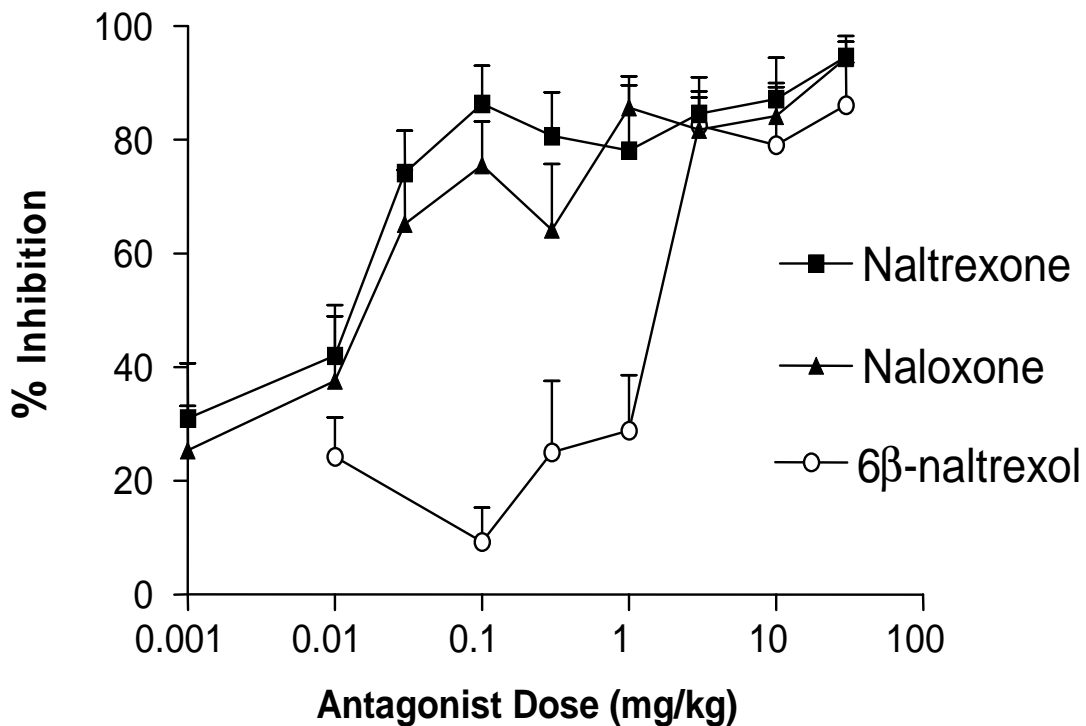


Figure 6.3. Percent inhibition (mean  $\pm$  SEM) data for naltrexone (■), naloxone (▲) and 6 $\beta$ -naltrexol (○) ( $n=10$ ) in antagonising morphine-induced (30 mg/kg) antinociception using the mouse hotplate test. Note: time interval between i) antagonist and morphine administrations = 15 min; ii) antagonist administration and test = 30 min

**6.4.2.2. Duration of Effect Potency Study**

Figure 6.4 summarises the duration of action of naltrexone, naloxone and 6 $\beta$ -naltrexol in antagonising the antinociceptive effects of morphine, using the highest dose of antagonist (30 mg/kg). There was no significant reduction in inhibition over the studied time-course. Figure  
*Susan J Porter PhD Thesis, 2010*

6.5 summarises the duration of naltrexone, naloxone and 6 $\beta$ -naltrexol when the IC<sub>50</sub>'s obtained for the acute study were administered over time. The morphine dose administered in both studies was 30 mg/kg, and the hotplate tests were conducted 30 minutes post-morphine administration. The time for the antagonist activity to decrease by 50% was 80 min for naltrexone, 120 min for naloxone, and 340 min for 6 $\beta$ -naltrexol.

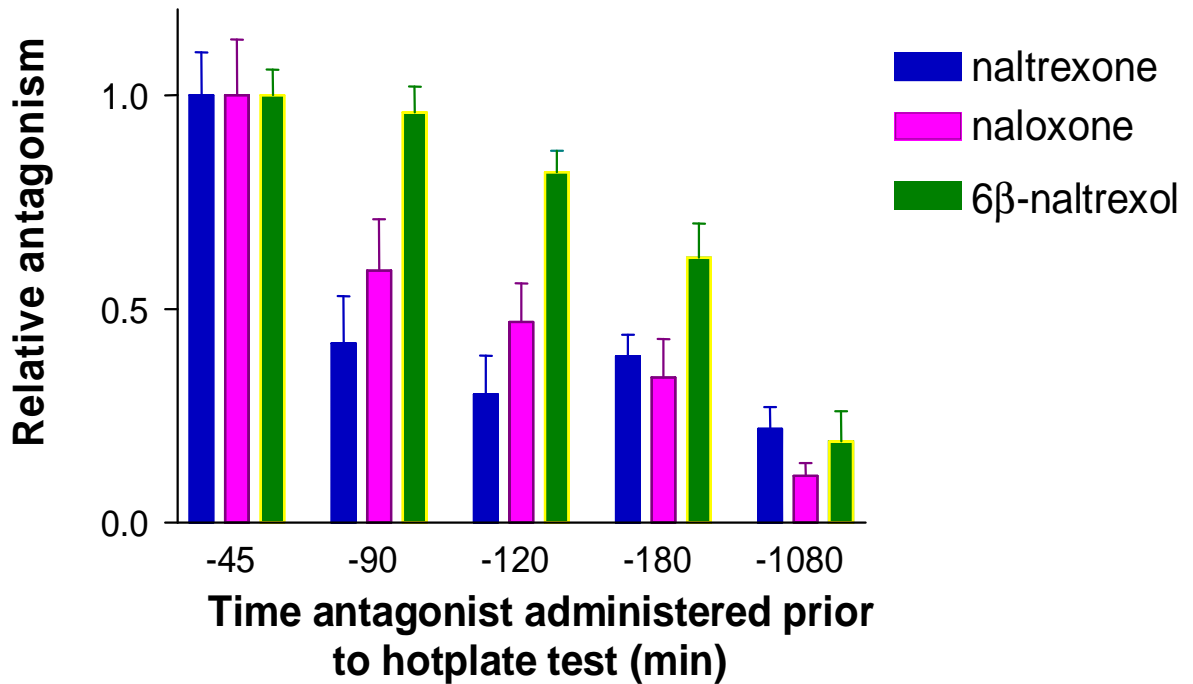


Figure 6.4. Mean  $\pm$  SEM ( $n=10$ ) duration of action of naltrexone, naloxone and 6 $\beta$ -naltrexone (all 30 mg/kg) on the antagonism of morphine (30 mg/kg) using the mouse hotplate test. Hotplate tests conducted 30 min post-morphine.

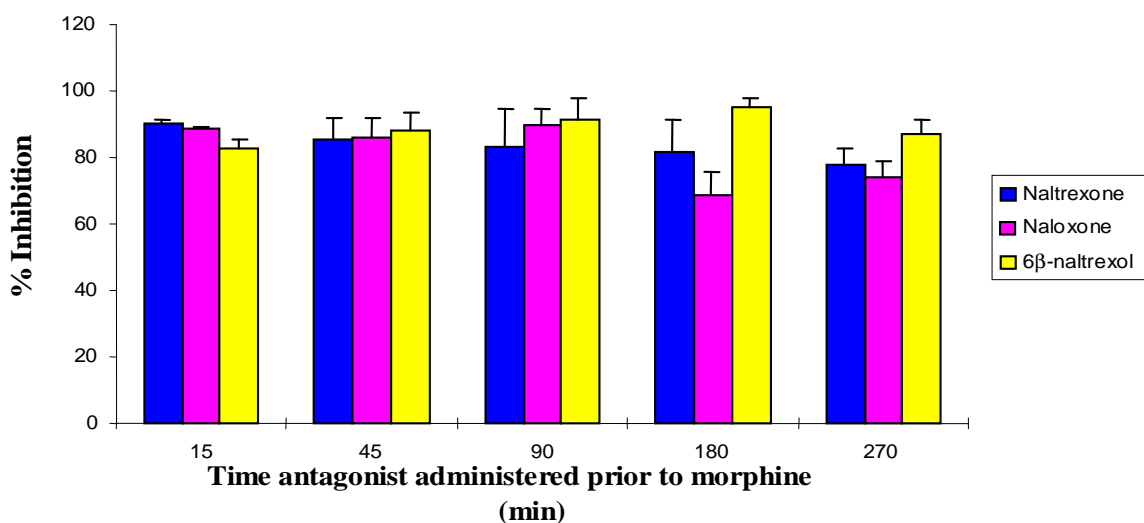


Figure 6.5. Mean  $\pm$  SEM ( $n=10$ ) duration of antagonism of morphine-induced (30 mg/kg) antinociception by naltrexone, naloxone and  $6\beta$ -naltrexol using the mouse hotplate test. Doses administered were: naltrexone 0.007 mg/kg, naloxone 0.016 mg/kg and  $6\beta$ -naltrexol 1.3 mg/kg.

### 6.5. Discussion

While there was a large variation in the data obtained for the *in vitro* potency study, the reasons for which are unknown the results obtained for naltrexone were similar to those previously reported (Takemori and Portoghese, 1984), whereby naltrexone was four times more potent than naloxone in preventing the morphine-induced reduction of twitch height of stimulated guinea pig ileum. In the current study,  $6\beta$ -naltrexol was 4.5-fold more potent than naloxone and 2.8-fold more potent than naltrexone.

The high potency of  $6\beta$ -naltrexol found in the *in vitro* study was not reflected in the acute *in vivo* mouse study, in which  $6\beta$ -naltrexol was only  $1/185^{\text{th}}$  as potent as naltrexone. This is similar to the potency value of  $1/85^{\text{th}}$  that of naltrexone found by Chatterjee and co-workers (1975) in counteracting oxymorphone-induced loss of righting reflex in rats; species differences and pharmacological response assessments might explain the differences between the two studies. In support of an earlier study (Blumberg and Dayton, 1974), naltrexone was approximately twice as potent as naloxone in the mouse in reversing the antinociceptive effects of morphine. In the present study, the  $ED_{50}$  values for naltrexone and naloxone were 7 and 16  $\mu\text{g}/\text{kg}$  respectively, whereas the earlier study by Blumberg and Dayton (1974) obtained  $ED_{50}$  values of 47 and 140  $\mu\text{g}/\text{kg}$ , respectively, in antagonising the oxymorphone-

induced Straub tail. The difference in ED<sub>50</sub> values between the two studies could indicate differences in the sensitivity between the Straub tail reaction and hotplate tests in mice in assessing antagonist potency.

In the current study, inhibition of antinociception by naltrexone calculated at 10 hours post-administration was less than 40% compared to greater than 40% inhibition at 10 hours post-administration previously reported by Alvarez-Fuentes and co-workers in mice (Alvarez-Fuentes et al., 2000). The longer duration of action observed in that study may be accounted for by the thousand-fold higher dose and controlled-release preparation used. The routes of administration were also different in the two studies, with the present study using intraperitoneal administration.

In humans, 6 $\beta$ -naltrexol remains in the systemic circulation much longer than naltrexone, irrespective of the route of administration (Wall et al., 1984). The terminal half-life values for the two compounds have been reported as ranging from 2-10 hours for naltrexone (McCaul et al., 2000a; Rukstalis et al., 2000; Wall et al., 1984), and 7.5-13 hours for 6 $\beta$ -naltrexol (McCaul et al., 2000a; Meyer et al., 1984; Wall et al., 1984). As reported in Chapter 5 above, elimination half-life values  $1.88 \pm 0.47$  hours and for 6 $\beta$ -naltrexol  $13.3 \pm 2.6$  hours were obtained. The longer half-life of 6 $\beta$ -naltrexol has been postulated as the reason for the longer duration of action of naltrexone compared to naloxone (half-life 60-90 min) in humans. While rodents do not produce 6 $\beta$ -naltrexol from naltrexone, or only in minute amounts (Malspeis et al., 1975), in the present study it was shown to have a much longer duration of action than either naltrexone or naloxone, which implies a longer half-life of 6 $\beta$ -naltrexol compared to naltrexone or naloxone.

Whereas the *in vivo* potency of 6 $\beta$ -naltrexol was shown to be much lower than the *in vitro* potency in the present study, factors such as metabolism, uptake and distribution into the central nervous system and receptor site concentrations must be taken into account when interpolating *in vivo* from *in vitro* data. The doses of 6 $\beta$ -naltrexol which were shown to antagonise morphine-induced antinociception by 80% in the present study (1-10 mg/kg), have been shown to significantly reduce alcohol consumption in the rat (Rukstalis et al., 2000), and this level of inhibition could be achieved by equipotent doses of naltrexone and 6 $\beta$ -naltrexol as shown in Figure 6.2.

While the potency of 6 $\beta$ -naltrexol was only 1/25<sup>th</sup> that of naltrexone in reducing ethanol

consumption in the rat (Stromberg et al., 2002), the serum concentrations of 6 $\beta$ -naltrexol are usually at least this much higher than naltrexone in humans after oral doses of naltrexone, therefore the data presented in this study support the role of 6 $\beta$ -naltrexol in the clinical effects of naltrexone.

The extensive first-pass metabolism of naltrexone after an oral dose (Meyer et al., 1984; Wall, 1981; this thesis Chapter 5) results in significantly higher blood concentrations of 6 $\beta$ -naltrexol compared with naltrexone. Therefore, while the *in vivo* potency of 6 $\beta$ -naltrexol is lower than that of naltrexone, the longer duration of action of 6 $\beta$ -naltrexol, and the significantly higher plasma concentrations of this metabolite after an oral dose of naltrexone indicate that 6 $\beta$ -naltrexol will contribute to the therapeutic and adverse effects of naltrexone. In addition, while 6 $\beta$ -naltrexol is less lipophilic than naltrexone and naloxone, with an octanol:water of 2.20 at pH 7.4 (Chapter 5), compared to 9.65 and 24.3 for naltrexone and naloxone, respectively at the same pH (Chapter 5), this is significantly higher than morphine, which is known to cross the blood:brain barrier to exert its central effects. The comparative octanol:water for morphine has been shown to be in the range of 0.56-0.63 at pH 7.4 (Chapter 5) (Van Crugten, 1991).

Evidence indicates that administration of 6 $\beta$ -naltrexol itself reduces alcohol consumption in rats (Rukstalis et al., 2000). Using a limited-access procedure, ethanol consumption was measured after rats (n=9) were non-randomly administered single doses of 6 $\beta$ -naltrexol of 2.5, 7.5, 12.5 and 25 mg/kg or a saline control. The authors measured the per cent decrease in ethanol consumption compared to baseline (7.4 g/kg/hr) and showed a significant reduction for the three higher 6 $\beta$ -naltrexol doses, with no change in water consumption. More recently, these researchers have compared the effects of 6 $\beta$ -naltrexol and naltrexone on the consumption of ethanol and sucrose using a similar limited-access ethanol paradigm (Stromberg et al., 2002). Using naltrexone doses of 0.1, 1.0 and 10 mg/kg and 6 $\beta$ -naltrexol doses of 15, 25 and 50 mg/kg, both of these drugs were shown to significantly reduce ethanol consumption over four days compared to saline control. In addition, 6 $\beta$ -naltrexol (25 mg/kg) and naltrexone (1.0 mg/kg) significantly reduced sucrose (10%) intake after an acute dose. Based on these data, they calculated the ED<sub>50</sub> values to be in the range of 25 mg/kg for 6 $\beta$ -naltrexol and 1.0 mg/kg for naltrexone for reducing ethanol consumption, indicating that naltrexone is 25 times more potent than 6 $\beta$ -naltrexol in this model.

In humans, McCaul and colleagues demonstrated that after administration of 50 and 100 mg of

naltrexone to heavy drinkers, there was a positive correlation between blood 6β-naltrexol concentrations and subjective feelings of sedation. Additionally, 16 hours after administration of 100 mg of naltrexone, a negative correlation was found between blood 6β-naltrexol concentrations and the subjects' self-reported feelings of pleasure after a high alcohol challenge (McCaul et al., 2000b).

The ability of 6β-naltrexol itself to reduce alcohol consumption in rats (Rukstalis et al., 2000; Stromberg et al., 2002), and the negative correlation between plasma 6β-naltrexol concentrations and subjective responses to alcohol (King et al., 1997), supports the influence of 6β-naltrexol in the effects of naltrexone. Thus 6β-naltrexol is likely to play an important role in the efficacy and patient compliance of naltrexone in clinical practice.

Since the completion of these studies, numerous studies have been conducted based on the premise that antagonists can be classified as inverse agonists and neutral antagonists. Inverse agonists can not only block the effects of an agonist at the receptor, but can also block the basal activity of the receptor in the absence of an agonist, which is increased with chronic agonist stimulation. Naltrexone is an inverse agonist, whereas 6β-naltrexol is a neutral antagonist (no effect on basal activity). Recent studies have been conducted to ascertain if the antagonist potencies of naltrexone and metabolites are altered in agonist-dependent compared to agonist-naïve animals, using various paradigms of antagonism.

In one such study, antagonistic potencies of naltrexone, 6β-naltrexol and naloxone (inverse agonist) were compared in opioid-naïve and opioid-dependent mice with respect to antinociception (tail-flick) assay, locomotor activity and precipitation of withdrawal (Raehal, 2005). The rank order of potency in the tail-flick assay was naltrexone > 6β-naltrexol > naloxone (icv) and naltrexone > 6β-naltrexol=naloxone (ip) (similar to those reported in this chapter for hot-plate assay). In another part of the study, various dosing regimens of morphine were used to induce mild, moderate or severe opioid dependence. Withdrawal was precipitated with naltrexone (0.1 mg/kg ip) or 6β-naltrexol (1 mg/kg ip). These authors reported a decreased severity of withdrawal with precipitation by 6β-naltrexol, and concluded that if the mouse results are applicable to humans, 6β-naltrexol, as a neutral antagonist, may be a better alternative to naltrexone or naloxone in reversing life-threatening effects of opioid overdose (by inducing less severe withdrawal profiles, and may be better tolerated in people recovering from severe dependence (Raehal, 2005).

## 7. Overall Discussion

### 7.1. Synthesis of 6 $\beta$ -naltrexol from naltrexone

The reduction of naltrexone hydrochloride using thiourea dioxide in an alkaline medium was successful and sufficient yields were obtained to conduct all further experiments. The products obtained did contain some 6 $\alpha$ -naltrexol (approximately 3%), which was insufficient to have any effect in the potency studies, but was evident in HPLC standard preparations as a small peak, which did not co-elute or cause interference with the naltrexone, 6 $\beta$ -naltrexol or naloxone peaks where present. There was no naltrexone in the final product as shown by infrared, mass spectrometry, proton NMR or HPLC.

### 7.2. The *in vitro* metabolism of naltrexone

This study showed that the hepatic enzymatic formation of 6 $\beta$ -naltrexol from naltrexone in human liver was confined to the cytosolic and not the microsomal fraction, exhibited considerable intersubject variability, and that the enzyme(s) involved could be inhibited by a number of compounds. The most potent inhibition was by the steroids testosterone, dihydrotestosterone and corticosterone, while the opioid antagonist naloxone also showed significant inhibition.

In the cytosol preparations variability was seen in both the  $V_{\max}$  (2.9-fold) and  $K_m$  (3.2-fold) values for 6 $\beta$ -naltrexol formation. This degree of variability has been shown in kinetic studies using enzymes from the cytochrome P450 family and the transferases. The intrinsic clearance ( $Cl_{\text{int}}$ ) values, which can be related to the *in vivo* intrinsic clearance of naltrexone suggest a drug of high hepatic extraction and showed an even larger variation (7.7-fold).

The data obtained in the present study are consistent with ketone reductase involvement as menadione, a known inhibitor of ketone-reducing enzymes (Wermuth, 1981), significantly inhibited the formation of 6 $\beta$ -naltrexol. Additionally, 6 $\beta$ -naltrexol formation was not detected in the microsomal fraction previously shown to catalyse CYP reactions, under conditions used frequently in this laboratory for a number of substrates. In the present study, there was no back-formation of 6 $\beta$ -naltrexol to naltrexone in the microsomal preparation tested.

### 7.3. Quantification of naltrexone and 6 $\beta$ -naltrexol in fluids

The method reported in this study is an easy procedure for the quantification of 6 $\beta$ -naltrexol in cytosolic preparations using HPLC with UV detection. I also developed methods for the

simultaneous quantification of naltrexone and 6 $\beta$ -naltrexol in a number of body fluids. These can be performed with good sensitivity and specificity using a simple extraction procedure and HPLC with electrochemical detection.

The assay for the quantification of 6 $\beta$ -naltrexol in cytosol using UV detection and the assays for the quantification of both naltrexone and 6 $\beta$ -naltrexol in plasma, blood, saliva and urine using electrochemical detection all had high levels of accuracy and precision. The only problems encountered related to the high concentrations of 6 $\beta$ -naltrexol in both hydrolysed and unhydrolysed urine samples, which necessitated an adaptation of the original method, and the binding of 6 $\beta$ -naltrexol to a constituent of the plasma, which necessitated the precipitation of plasma proteins with acetonitrile prior to extraction in order to obtain a recovery of 6 $\beta$ -naltrexol greater than 90%.

Based on the results of the quality control samples, the plasma assays for naltrexone and 6 $\beta$ -naltrexol continued to perform with a high degree of accuracy and precision. The quantification assays of naltrexone and 6 $\beta$ -naltrexol in urine also remained high in accuracy and precision for all quality control samples tested. The ongoing performance of the high-concentration urine assays was also highly accurate and precise. The ongoing performance of the saliva assay showed accuracy of >80% for naltrexone and 6 $\beta$ -naltrexol, with coefficients of variation of <12% for both analytes.

#### **7.4. Human Pharmacokinetic studies**

In the current study, the plasma concentrations of naltrexone after an intravenous dose of 1 mg were quantifiable only up to 4 hours post-dose in all but one of the volunteers; this person had detectable levels to 12 hours. Plasma levels of 6 $\beta$ -naltrexol were undetectable after intravenous naltrexone. The major urinary metabolites observed after the IV dose were unconjugated 6 $\beta$ -naltrexol and conjugated naltrexone and 6 $\beta$ -naltrexol. The total percent of the dose excreted as naltrexone and 6 $\beta$ -naltrexol in 96 hours after one mg of IV naltrexone was 49%. There was a large interindividual variation in the excretion profiles of naltrexone and 6 $\beta$ -naltrexol.

Following a 50 mg oral dose of naltrexone, the maximum concentrations of naltrexone and 6 $\beta$ -naltrexol were obtained 0.9 and 0.8 hours, respectively, post naltrexone dose. The  $C_{\max}$  concentrations of naltrexone after an oral dose of 50 mg were  $8.0 \pm 6.7$  ng/ml and, like the



urinary excretion data, there was large variation in results. The maximum plasma 6 $\beta$ -naltrexol concentrations obtained were  $127 \pm 36$  ng/ml, and were higher than those of naltrexone at all time points following the oral dose of naltrexone. The mean plasma half-life value for 6 $\beta$ -naltrexol ( $13.3 \pm 2.6$  hr) was significantly longer than that for naltrexone ( $1.88 \pm 0.47$  hr). The AUC values for 6 $\beta$ -naltrexol were  $39 \pm 15.5$  times higher than naltrexone, and the IV and oral data indicate that naltrexone is almost completely absorbed, has a high first-pass biotransformation and low oral bioavailability (26%). The naltrexone clearance data show a total clearance greater than that of liver blood flow, and as little of the drug is excreted unchanged in the faeces, there must be considerable extrahepatic clearance. Renal clearance accounts for only a small proportion of total clearance, while biotransformation accounts for a larger proportion; the parent drug could be metabolised to unmeasured metabolites.

The major urinary excretion products obtained following administration of the oral dose of naltrexone (50 mg), were conjugated and unconjugated 6 $\beta$ -naltrexol, followed by conjugated naltrexone. The naltrexone was excreted mostly as the glucuronide conjugate whereas the 6 $\beta$ -naltrexol was excreted mostly unconjugated. The urinary excretion of both products accounted for only 35% of the naltrexone dose administered. Part of the rest of the dose could be in the form of 2-hydroxy-3-methoxy-6 $\beta$ -naltrexol (as reported by Verebey *et al*, 1975), and 2-hydroxy-3 methoxy-naltrexone (as reported by Cone *et al*, 1978). As these are only minor metabolites (no more than 8% of dose if present), and as faeces accounts for no more than 5%, there is still a substantial proportion of the dose unaccounted for, which could be as a metabolite as yet unidentified.

The degree of plasma protein binding for naltrexone was found to be low at 15% and the plasma binding of 6 $\beta$ -naltrexol following an oral dose of naltrexone in the current study was significantly higher than that of naltrexone, at 26%.

Both naltrexone and 6 $\beta$ -naltrexol were undetectable in saliva after an IV naltrexone dose of one mg. However, following the 50 mg oral dose, the salivary concentrations were lower than those seen in the urine, but higher than those measured in the plasma. The peak naltrexone concentrations in saliva ranged from 74-249 ng/ml (mean  $C_{\max}$   $164 \pm 59$ ) and the peak 6 $\beta$ -naltrexol concentrations ranged from 209-455 ng/ml (mean  $C_{\max}$   $323 \pm 114$ ). The elimination half-life values for naltrexone ranged from 2.5 to 3.3 hours, mean  $2.9 \pm 0.3$  hours

and from 14.5 to 37.4 hours for 6 $\beta$ -naltrexol, with a mean of  $20.8 \pm 8.4$  hours. The salivary pH did not have any effect on naltrexone or 6 $\beta$ -naltrexol concentrations. Saliva:plasma AUC values were  $19.5 \pm 8.3$  and  $3.5 \pm 1.2$  for naltrexone and 6 $\beta$ -naltrexol, respectively. In conclusion, the results show that there is large variation in the pharmacokinetic parameters tested between individuals, and that saliva is possibly a better alternative to plasma in assessing naltrexone status following the 50 mg daily dose used clinically.

At the doses given to volunteers in the current study (1 mg IV and 50 mg oral), there were no reported subjective side-effects with the exception of increased confusion/bewilderment following the IV dose compared to the oral dose, but this effect was present at the baseline sample, so is unlikely to be a drug effect. There was also no correlation between concentrations of naltrexone and 6 $\beta$ -naltrexol in plasma, urine or saliva and subjective side effects.

#### **7.5. *In vitro* and *in vivo* potency studies**

In the present *in vitro* study naltrexone was four times more potent than naloxone in preventing the morphine-induced reduction of twitch height of stimulated guinea pig ileum, whereas 6 $\beta$ -naltrexol was 4.5-fold more potent than naloxone and 2.8-fold more potent than naltrexone in this model.

The high potency of 6 $\beta$ -naltrexol found in the *in vitro* study was not reflected in the acute *in vivo* mouse study, in which 6 $\beta$ -naltrexol was only 1/185<sup>th</sup> as potent as naltrexone. Naltrexone was approximately twice as potent as naloxone in the mouse in reversing the antinociceptive effects of morphine. The ED<sub>50</sub> values for naltrexone and naloxone and 6 $\beta$ -naltrexol were 0.007, 0.016 and 1.30 mg/kg respectively. Using these ED<sub>50</sub> values, the time for the antagonist activity to decrease by 50% was 80 min for naltrexone, 120 min for naloxone, and 340 min for 6 $\beta$ -naltrexol.

While the *in vivo* potency of 6 $\beta$ -naltrexol was shown to be much lower than the *in vitro* potency in the present study, factors such as metabolism, uptake and distribution into the central nervous system and receptor site concentrations must be taken into account when interpolating *in vivo* from *in vitro* data. The doses of 6 $\beta$ -naltrexol which were shown to antagonise morphine-induced antinociception by 80% in the mouse ranged from 1-10 mg/kg, Blumberg and Dayton (1976) obtained ED<sub>50</sub> values of 47 and 140  $\mu$ g/kg, respectively, in

antagonising the oxymorphone-induced Straub tail. The difference in ED<sub>50</sub> values between the two studies could indicate differences in the sensitivity between the Straub tail reaction and hotplate tests in mice in assessing antagonist potency.

Rodents do not produce 6 $\beta$ -naltrexol from naltrexone, or only in minute amounts (Malspeis et al., 1975). Therefore, the longer duration of action compared to naltrexone or naloxone was not due to metabolism of naltrexone, and implies a longer half-life of 6 $\beta$ -naltrexol compared to naltrexone or naloxone. It is expected that 6 $\beta$ -naltrexol would cross the blood-brain barrier as although it is less lipophilic (octanol:water 3.1) than naltrexone and naloxone (octanol:pH7.4 buffer 10.9 and 26.4, respectively), it is significantly more lipophilic than morphine (octanol: pH7.4 buffer 0.56), which is known to cross the blood:brain barrier to exert its central effects.

It has been proposed that  $\mu$ -opioid receptors exhibit a constitutively active form, and that naltrexone as an inverse agonist can block this basal activity, whereas 6 $\beta$ -naltrexol has no effect on basal activity. It has been hypothesised that an inverse opioid receptor agonist that decreases basal signalling of a constitutively active receptor should precipitate a more severe withdrawal response than a neutral antagonist which can only precipitate withdrawal by displacement of an agonist, and that these differences are due to an apparent difference in their *in vivo* receptor antagonist potencies caused by differential access to agonist-occupied receptors. In this study, naltrexone and 6 $\beta$ -naltrexol antagonism of morphine-induced antinociception, time-course of antagonism of a long-acting agonist (BU72), and precipitation of withdrawal in morphine-dependent mice were assessed and compared. The results of these studies led the authors to conclude that while these antagonists have equivalent *in vivo* receptor affinities, the differences observed in efficacy could be due to different abilities in reaching the site of action (central  $\mu$ -opioid receptors) and the rapidity of access needed to precipitate a robust withdrawal response (Divin, 2008). The ability of antagonists to act as inverse or neutral agonists could explain the results reported in the current study (chapter 6), with respect to the relative potencies of naltrexone, naloxone and 6 $\beta$ -naltrexol, and the fact that the differences in relative potencies were very different in the *in vitro* preparations.

## 7.6. Conclusions

The results reported in the studies described above, provide further evidence for the major human metabolite of naltrexone 6 $\beta$ -naltrexol being active, and very likely to contribute substantially to

the opioid antagonist effects when naltrexone is given. The *in vitro* potency of the metabolite was higher in the guinea pig ileum preparation than the parent compound, but the *in vivo* potency of 6 $\beta$ -naltrexol in the mouse model was not as high as that of naltrexone, but had a much longer duration of action. Therefore, it is likely that the therapeutic effects of naltrexone are due in part to the action of 6 $\beta$ -naltrexol.

While the assay used in the current study for the quantification of naltrexone is not as sensitive as some of those described earlier (Davidson et al., 1996; Meyer et al., 1984; Reuning et al., 1989; Wall et al., 1984), the use of radiolabelled compounds was avoided in the current study and the samples did not have to undergo complicated extraction and derivatisation procedures and smaller sample volumes (1.0 ml) were needed. Additionally, with the exception of very high concentrations in hydrolysed urine samples, both naltrexone and 6 $\beta$ -naltrexol can be assayed simultaneously.

The reduction of naltrexone to 6 $\beta$ -naltrexol was shown to be cytosolic in the current study, and could be significantly inhibited by the anabolic steroids testosterone and dihydrotestosterone. Any factor that decreases or increases the amount of 6 $\beta$ -naltrexol formed, or its elimination will affect plasma 6 $\beta$ -naltrexol concentrations and will have subsequent effects on the antagonism of opioid agonists.

The potent inhibition by androgenic steroids could cause further variability in naltrexone efficacy and contribute to adverse effects. Conversely, the effect of naltrexone treatment on levels of circulating steroid hormones in both males and females is unknown, and long-term studies in animals to assess any changes to steroidogenesis are warranted. The large partial hepatic intrinsic clearance is consistent with naltrexone having a high hepatic first pass when given orally. While naltrexone metabolism appears to be unaffected by the presence of likely co-administered drugs, the pharmacokinetic factors affecting the formation and elimination of 6 $\beta$ -naltrexol could influence patients' willingness to remain on naltrexone treatment, and this needs to be investigated.

The large variation found in the cytosolic preparations for the reduction of naltrexone could also account, in part, for the inter-individual variation found in this and previous pharmacokinetic studies of naltrexone. The findings of King *et al.*, (1997), that there was a correlation of subjective side-effects and high urinary 6 $\beta$ -naltrexol concentrations, was not reproduced in this study, but this may be a result of the low number (6) of volunteers in the present study.

However, the much longer half-life of 6 $\beta$ -naltrexol, the much higher concentrations present in

plasma, saliva and urine, together with the much longer duration of action suggest that, therapeutically, it is likely that 6 $\beta$ -naltrexol will be the active antagonist in the body, with much less contribution from naltrexone. It is likely also that the side effects reported for naltrexone are also due mostly to the high levels of 6 $\beta$ -naltrexol in the circulating plasma. Further studies examining the concentrations of naltrexone and 6 $\beta$ -naltrexol in the brain could help elucidate the likely contributions of each analyte to the effects, therapeutic or otherwise of naltrexone.

In conclusion, the current study has extended our knowledge of the metabolism of naltrexone, and more particularly the actions and contribution of the major human metabolite of naltrexone, 6 $\beta$ -naltrexol.

**Appendix 1: Publications in Support of this Thesis**

Porter, S.J., Somogyi, A.A. and White, J.M. (2000) Kinetics and inhibition of the formation of 6 $\beta$ -naltrexol from naltrexone in human liver cytosol.  
*British Journal of Clinical Pharmacology*, v. 50 (5), pp. 465-471, November 2000

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1046/j.1365-2125.2000.00281.x>

Porter, S.J., Somogyi, A.A. and White, J.M. (2002) In vivo and in vitro potency studies of 6 $\beta$ -naltrexol, the major human metabolite of naltrexone. *Addiction Biology*, v. 7 (2), pp. 219-225, April 2002

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

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