

Université de Montréal

A Systematic In Vitro Metabolism Study of Opioids using Rat Liver S9
Fractions and Mass Spectrometry Revealed CYP2D Metabolism is
Impaired with age

par

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Mémoire présenté en vue de l'obtention du grade de maître ès sciences (M.Sc.) en
sciences vétérinaires option pharmacologie

Juillet 2016

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Résumé

La codéine et l'oxycodone sont des opioïdes utilisés pour soulager la douleur. Le succès du traitement dépend du métabolisme par les enzymes CYP450. Selon les médicaments utilisés, l'altération de métabolisme peut entraîner de graves conséquences, y compris des modifications dans leurs profils d'efficacité, de sécurité et de toxicité. L'objectif de cette étude consistait à évaluer par HPLC-MS / MS (trappe ionique linéaire) l'effet de l'âge sur le métabolisme de l'enzyme CYP2D chez les rats. La codéine et l'oxycodone ainsi que leurs métabolites spécifiques, morphine (CYP2D), norcodeine (CYP3A), oxymorphone (CYP2D) et noroxycodone (CYP3A), ont été quantifiés en utilisant la méthode de dilution isotopique. Les fractions S9 de foie de rats (Sprague-Dawley) de sexe masculin âgés de 3, 6, 12 et 18 mois ont été préparées et les paramètres de Michaelis-Menten ont été déterminés pour les principales voies métaboliques. La vitesse maximale de la réaction enzymatique (V_{max}) a montré une saturation rapide des sites actifs de CYP2D dans la fraction s9 de foie chez les rats âgés de 18 mois. Les valeurs de V_{max} observées pour la codéine chez les rats âgés de 3, 6 et 12 mois étaient respectivement de $0,327 \pm 0,027$, $0,355 \pm 0,021$ et $0,273 \pm 0,028$. Concernant l'oxycodone, les valeurs étaient de $0,690 \pm 0,096$, $0,499 \pm 0,087$, $0,153 \pm 0,01$ respectivement. Cependant, la valeur de V_{max} chez les rats âgés de 18 mois était de $0,092 \pm 0,003$ pour la codéine et de $0,153 \pm 0,01$ pour l'oxycodone. Vraisemblablement, la valeur de V_{max} affecte de manière significative ($P \leq 0,05$) la clairance enzymatique de ces médicaments ($CL_{uint} = V_{max} / K_m$). Les résultats de cette étude suggèrent que l'enzyme CYP2D subit un changement conformationnel avec l'âge, particulièrement chez les rats âgés de 18 mois. Ce changement conformationnel conduit à une diminution significative du taux de formation des métabolites opioïdes actifs, ce qui entraîne une diminution de la clairance enzymatique de ces médicaments. En conclusion, cette étude suggère une détérioration des CYP2D et CYP3A chez les rats vieillissants, ce qui affecte le métabolisme des médicaments.

Mots-clés: Opioïdes, vieillissement, métabolisme des médicaments, CYP450, CYP2D, CYP3A, spectrométrie de masse.

Abstract

Codeine and oxycodone are opioids used in the treatment of pain. The outcome of the treatment is ultimately related to their metabolism by CYP450 enzymes. Depending on the drugs used, alterations in the metabolism of drugs by CYP450 enzymes can lead to severe consequences including alterations in their efficacy, safety and toxicity profiles. The objective of this study was to assess the effect of age on CYP2D enzyme metabolism in rats using HPLC-MS/MS (Linear Ion Trap). Codeine and oxycodone along with specific metabolites morphine (CYP2D), norcodeine (CYP3A), and oxymorphone (CYP2D), noroxycodone (CYP3A) were quantified using isotopic dilution method. Liver S9 fractions from 3, 6, 12, and 18 month-old male Sprague Dawley rats were prepared and Michaelis-Menten parameters were determined for their primary metabolic pathways. The derived maximum enzyme velocity (V_{\max}) suggested a rapid saturation of the CYP2D active sites in the liver S9 fractions of 18 month-old rats. The observed V_{\max} values for codeine in 3, 6 and 12 month-old rats respectively were 0.327 ± 0.027 , 0.355 ± 0.021 and 0.273 ± 0.028 and for oxycodone, the values were 0.690 ± 0.096 , 0.499 ± 0.087 , 0.153 ± 0.01 . For 18 month-old rats, V_{\max} value was 0.092 ± 0.003 for codeine compared to 0.153 ± 0.01 for oxycodone. The V_{\max} value affected significantly ($P \leq 0.05$) the enzyme-mediated clearance of these drugs ($CL_{\text{uint}} = V_{\max} / K_m$). The results of this study suggest that CYP2D enzyme undergoes a conformational change with age, particularly in 18 month-old rats considered to be geriatric in age, thus leading to a significant decrease in the rate of the formation of active opioid metabolites and a decrease in enzymatic clearance of these drugs. This study suggests that there is an impairment of CYP2D and CYP3A in aging rats, which affects their drug metabolism.

Keywords: Opioids, Aging, Drug metabolism, CYP450, CYP2D, CYP3A, Mass spectrometry.

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Abbreviations list

AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
C-6-G	Codeine-6-glucuronide
CL	Clearance
Cl _{uint}	Enzyme-mediated clearance
CYP450	cytochrome P450
DOR	δ-opioid receptor
EM	Extensive metabolizer
ER	Endoplasmic reticulum
FDA	Food and drug administration
GABA	γ-amino butyric acid
GDP	Guanosine diphosphate
GIT	Gastrointestinal tract
Gly	Glycine
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
IM-	Intermediate Metabolizer
i.m.	Intramuscular
i.v.	Intravenous
K _m	Michaelis-Menten constant
Leu	Leucine
KOR	k-opioid receptor
MOR	μ-opioid receptor
MS	Mass spectrometry
M-3-G	Morphine-3-glucuronide
M-6-G	Morphine-6-glucuronide
NADPH	Nicotinamide adenine dinucleotide phosphate
OR	Opioid receptor

ORL-1	Opioid-like receptor 1
Phe	Phenylalanine
PM	Poor metabolizer
Tyr	Tyrosine
UDPGA	Uridine 5'-diphospho-glucuronic acid
UGT	Uridine 5'-diphospho-glucuronyltransferase
UM	Ultra-rapid metabolizer
V_{\max}	maximum velocity of metabolic reaction

Acknowledgment

I like would to thank my supervisor Dr Francis Beaudry for giving me the opportunity to pursue a master degree under his supervision. His patience, understanding and perseverance acted as my guiding light. A heartfelt thank you to him for pointing out my weaknesses and helping me to overcome them.

I extend my initial gratitude to my supervisory committee members Younes Chorfi and a later member Dr Kalidou Ndiaye who helped me in my analytical and research thinking and never failed to advise me for my own betterment. A special thanks to Pascal Vaschon and Marie-Chantal Giroux for the work they did in collecting tissue samples for this project and to the Canadian Association for Laboratory Animal Sciences for their funding.

Finally thank you to Professor Amer Silim and his wife Mrs Carol Jones for they are the reason I was able to come and pursue my masters studies at the Université de Montréal.

Last but not least I am grateful to my parents for allowing me to pursue my post graduate studies in a country so far away from home and to my friends and colleagues for the constant support, help and encouragement.

Introduction

The recent advancement in the past decade in the medical field has led to an increase in the average life expectancy. In 2013, a survey showed that 14.1% of the US population are above the age of 65 and this is expected to increase to 21.7% by 2040 (Ortman *et al.*, 2014). Elderly patients suffer from pain due to reasons, which include but are not limited to diseases. To improve the quality of life of these patients, they are prescribed medications for pain relief and life style changes that include eating healthy, losing a small amount of weight and increasing physical activity.

Opioids are considered first-line therapy for pain relief. They are widely used for the management of all types of pain ranging from mild pain such as backache to severe pain such as post-surgical pain or cancer pain. The pharmacokinetics and pharmacodynamics of opioids are known to be altered with aging (Mézière *et al.*, 2013; Woodhouse & Wynne, 1988). Extensive research and literature reviews are available on this subject and some of the widely accepted explanations include decline in lean body mass, decrease in hepatic blood flow and disease conditions. In addition, studies suggest a decreased hepatic metabolism and renal clearance of drugs in elderly patients due to physiological aging or the presence of diseases like renal failure, hepatic failure and cardiovascular complications (Mangoni & Jackson, 2004). The reduction in hepatic clearance with age has not been satisfactorily elucidated and remains to some extent controversial.

A large number of drugs are metabolized in the liver by CYP450 enzymes to active or inactive metabolites. The efficacy of the drugs bioactivated by CYP450 enzymes and the clearance of drugs metabolized by these enzymes are affected by any change in the activity of these enzymes. The isoforms of CYP450: CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2C8 and CYP2B6 contribute greatly to opioid metabolism in the body. CYP3A4 and CYP2D6 are the primary metabolizers of a large proportion of opioid drugs. Alteration in the functionality of CYP3A4 and CYP2D6 with age leads to a change in the efficacy, safety, toxicity and side effects of these drugs.

Codeine and oxycodone are well-known substrates for CYP2D and CYP3A. Both drugs act on the μ -opioid receptor (MOR) in the CNS to cause analgesia. Codeine is metabolized by CYP2D6 to morphine, to which it owes its analgesic effect, and by CYP3A4 to noroxycodone, an inactive metabolite. Oxycodone is metabolized to oxymorphone, an active metabolite and noroxycodone, an inactive metabolite by CYP2D6 and CYP3A enzymes respectively. In rats, it has been shown that CYP2D6 corresponds to CYP2D1 and CYP3A4 corresponds to CYP3A2 in terms of structure and function (Martigoni *et al.*, 2006). Previous studies have shown that CYP3A is impaired in 18-month old rats leading to a decreased enzymatic clearance of ketamine (Santamaria R. *et al.* 2015).

In general, drugs present in the pharmaceutical markets have not been sufficiently tested in geriatric and pediatric patients due to ethical considerations and regulatory restrictions. An understanding of factors that influence drug metabolism is important for a safe and effective drug use in the elderly. Enzyme modification can have a significant impact on the formation and elimination of active and inactive metabolites thus resulting in a profound effect on the pharmacological and toxicological outcomes.

The aim of this research project was to study the effect of age in rats on the impairment of drug metabolism by CYP2D enzyme which can be due to a decrease in the metabolic function of the enzyme with age hence leading to a decrease in active metabolite formation and accumulation of parent drug in the body. In this study, rat liver S9 fractions from 3, 6, 12 and 18 month-old rats were used to evaluate the effect of aging on metabolic stability and enzyme-mediated clearance by CYP2D and CYP3A, based on Michaelis-Menten approach for specific metabolic pathways. Codeine and oxycodone were used to characterize functional changes since they are well-defined substrates for CYP2D and CYP3A functionality. HPLC-MS method was used to quantify codeine, norcodeine, morphine, oxycodone, oxymorphone and noroxycodone using isotopic dilution strategy in liver S9 fraction suspension.

I LITERATURE REVIEW

I.1 Historical View of Opioids

Around 3400 BC the Sumerians on Mesopotamia were among the first people to have cultivated the poppy plant, *Papaver somniferum*. They called it Hul Gil, the ‘joy plant’. Eventually the plant spread throughout the ancient world to every civilization in Europe and Asia and it was used to treat pain and many other ailments (Smith, 2009).

The poppy plant was cultivated in the ancient civilizations of Persia, Egypt and Mesopotamia. Archaeological evidence and fossilized poppy seeds suggest that Neanderthal man may have used the opium poppy over thirty thousand years ago. Less controversially, the first known written reference to the poppy appears in a Sumerian text dated around 4,000 BC. *Papaver somniferum* has long been popular in Europe. Fossil remains of poppy-seed cake and poppy-pods have been found in Neolithic Swiss lake-dwellings dating from over 4,000 years ago. Poppy images appear in Egyptian pictography and Roman sculpture and opium could readily be bought on the street-markets of Rome. By the eighth century AD, opium use had spread to Arabia, India and China. The Arabs both used opium and organized its trade.

A significant advance in opium-processing occurred in the sixteenth century. In freebase form, the alkaloids found in opium are significantly less soluble in water than in alcohol. Philippus Aureolus Theophrastus Bombastus von Hohenheim (1490-1541), better known as Paracelsus, claimed: "I possess a secret remedy which I call *laudanum* and which is superior to all other heroic remedies". He concocted laudanum [literally: "something to be praised"] by extracting opium using brandy, thus producing, in effect, tincture of morphine. Laudanum remained largely unknown until the 1660 when English physician Thomas Sydenham compounded a proprietary opium tincture that he also called laudanum, although it differed substantially from laudanum by Paracelsus. By the nineteenth century, vials of laudanum and raw opium were freely available at any English pharmacy or grocery store.

Until the nineteenth century, the only opioids used medicinally or recreationally took the form of crude opium. Opium is a complex chemical cocktail containing sugars, proteins, fats, water, meconic acid, plant wax, latex, gums, ammonia, sulphuric and lactic acids, and numerous

alkaloids, most notably morphine (10%-15%), codeine (1%-3%), noscapine (4%-8%), papaverine (1%-3%), and thebaine (1%-2%). All of the latter, apart from thebaine, are used medicinally as analgesics. The opioid analgesics are of inestimable value because they reduce or stop pain without causing a loss of consciousness. They also relieve coughs, spasms, fevers and diarrhea.

Even thebaine, though without analgesic effect, is of immense pharmaceutical value. This is because it can be used to produce semi-synthetic opioid morphine analogues such as oxycodone (Percodan), dihydromorphenone (Dilaudid), hydrocodone (Vicodin) and etorphine (Immobilon). Classes of morphine analogue include diphenylpropylamines (e.g. methadone), 4-phenylpiperidines (e.g. meperidine), morphinans (e.g. levorphanol) and 6,7-benzomorphans (e.g. metazocine). Although seemingly structurally diverse, all these compounds either possess a piperidine ring or contain the critical part of its ring structure (Williams, 2011).

Morphine was first isolated from opium in 1805 by a German pharmacist, Wilhelm Sertürner, who described it as the *Principium somniferum*. He named it morphium after Morpheus, the Greek god of dreams. Doctors had long hunted for effective ways to administer drugs without ingesting them. Taken orally, opium is liable to cause unpleasant gastric side effects. The development of the hypodermic syringe in the mid-nineteenth century allowed the injection of pure morphine, after which it started being used for minor surgical procedures, for post operative and chronic pain and as an adjunct to general anesthetics. In 1874, English pharmacist C.R. Alder Wright boiled morphine and acetic acid to produce diacetylmorphine. Diacetylmorphine was then synthesized and marketed commercially by the German pharmaceutical company, Bayer and in 1898, Bayer launched heroin the best-selling drug-brand of all time made from morphine (Rassool, 2009).

Codeine, a less powerful drug that is found in opium but can be synthesized, was first isolated in 1830 in France by Jean-Pierre Robiquet, to replace raw opium for medical purposes. In 1874, chemists trying to find a less addictive form of morphine made heroin. But heroin had twice the potency of morphine, and heroin addiction soon became a serious problem. Methadone was first synthesized in 1937 by German scientists Max Bockmühl and Gustav Ehrhart at the IG Farben company. They were searching for a painkiller that would be easier to use during surgery, with less addiction potential than morphine or heroin. New painkillers that came to the market in the

USA with approval from the Food and Drug Administration includes Vicodin in 1984, OxyContin in 1995 and Percocet in 1999. These are all synthetic opiates which mimic the body's own painkillers.

Opioids are considered to be the standard care for the management of acute and chronic pain, but long-term administration of opioids for the treatment of chronic non-cancerous pain is controversial (Rosenblum *et al.*, 2008). The concerns related to the effectiveness, safety and abuse of opioids has led to two different approaches; the first one is a more restrictive perspective, whereas the second one is a greater willingness to endorse this treatment (Smith, 2009). It was only after the publication of reports on the safety and efficacy of opioids prescribed to small numbers of patients with chronic non-malignant pain and the publication of a seminal article entitled "The Tragedy of Needless Pain" that opioid prescription for chronic non-malignant pain began to be practiced.

I.2 Opioids

Opioids refer to compounds that bind to opioid receptors (OR) and exert their pharmacological or physiological effects on the body. An important effect of opioids is the reduction in the perception of pain. The human body has an innate pain relieving system made of endogenous opioids. Like their endogenous counter parts opioid drugs bind to OR to produce analgesia with some undesirable effects (Holden *et al.*, 2005).

I.3 Opioid receptors and their mechanism of action

OR and their respective genes have been characterized at cellular, molecular and pharmacological levels into four different OR systems μ -opioid receptor (MOR), κ -opioid receptor (KOR), δ -opioid receptor (DOR), and opioid receptors like-1 (ORL-1) (Al-Hasani & Bruchas, 2011). ORL-1 is genetically closely associated with the previous three receptors, but displays a pharmacological profile that greatly differs from MOR, KOR and DOR as ORL-1 does not bind opioid ligands (Connor & Christie, 1999; Mollereau & Mouldous, 2000). The MOR, KOR, DOR and ORL-1 receptors are encoded by OPRM1, OPRD1, OPRK1 and OPRL1 genes respectively. The OR genes are highly conserved at the sequence encoding the seven transmembrane fragment but they vary at the carboxyl and amino termini. The difference in

affinity to opioid ligands and the distinct signaling pathways of the OR is a result of this variation (Levrán *et al.*, 2012).

OR are expressed in the central nervous system and in the peripheral organs such as the heart, intestines, kidneys, lungs, liver and reproductive tracts (Abbadie *et al.*, 2004; Beadles-Bohling & Wiren, 2005; Mansour *et al.*, 1995; Villemagne *et al.*, 2002; Vogt *et al.*, 1995; Wittert *et al.*, 1996; Xia & Haddad, 1991, 2001). They are involved in a large number of physiological processes that include growth, reproduction, respiration, immunological response, physiology of the gastrointestinal tract (GIT) and pain signaling in the central and peripheral nervous system. The expression and distribution of OR vary among different organs and different species (Barry & Zuo, 2005).

OR have been identified on cell bodies in the dorsal root ganglion and on central terminals of primary afferent neurons within the dorsal horn of the spinal cord (Stein *et al.*, 1996). They have also been detected on peripheral nervous system on primary afferent neurons in animals (Coggeshall *et al.*, 1997; Hassan *et al.*, 1993; Li *et al.*, 1996; Mousa *et al.*, 2001; Stein *et al.*, 1990; Wenk & Honda, 1999) and in humans (Stein *et al.*, 1996). The binding characteristics of OR on primary afferent neurons were shown to be very similar to those in the brain by binding experiments (Hassan *et al.*, 1993).

In the pain-modulation descending pathway, OR are expressed in the medulla and the periaqueductal gray area. They are also expressed in the limbic, the midbrain and the cortical structures (Al-Hasani & Bruchas, 2011). The activation of OR at these locations directly inhibits neurons, which in turn inhibits spinal cord pain transmission (Ahlbeck, 2011; McNicol *et al.*, 2003).

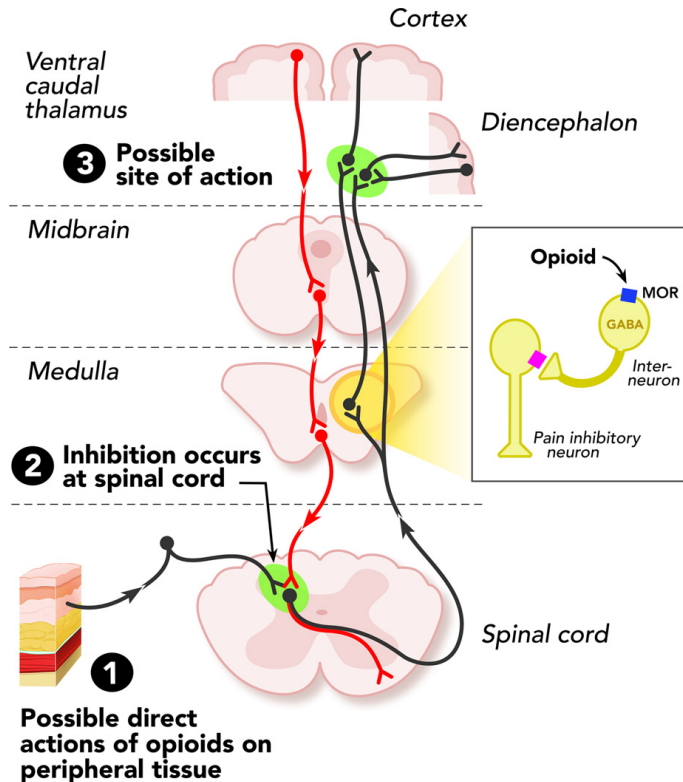


Figure 1: Sites of action of opioid analgesics. The ascending pathway shows the sites of central action on the pain transmission. The descending pathway shows the actions on pain modulating neurons in the mid brain and medulla. 1- Opioids can act directly on peripheral tissue. 2- Inhibition of pain by opioids can take place at the spinal cord. 3- Some opioids act directly on the brain to relieve pain. Adapted from Al-Hasani & Bruchas, 2011.

OR are G-protein-coupled receptors with seven transmembrane-spanning domains and are present on neuronal cell membranes. G-proteins consist of α , β and γ subunits. The α subunit is coupled with a guanosine diphosphate (GDP). When an agonist binds to an OR, it causes a conformational change leading to the attachment of guanosine triphosphate (GTP) to the α -subunit, which in turn dissociates it from the β - γ stable dimer and the receptor (Fig.2). Both the α -subunit and β - γ stable dimer can modulate several cellular signaling pathways (Childers *et al.*, 1979; Childers & Snyder, 1978; Gether, 2000). These include among others, stimulation or inhibition of adenylate cyclase (Gether, 2000; Minneman & Iversen, 1976), activation of phospholipases and regulation of potassium and calcium channels. This complex along with the β and γ group subsequently act on different intracellular pathways (Childers *et al.*, 1979; Childers & Snyder, 1978) (Fig.2)

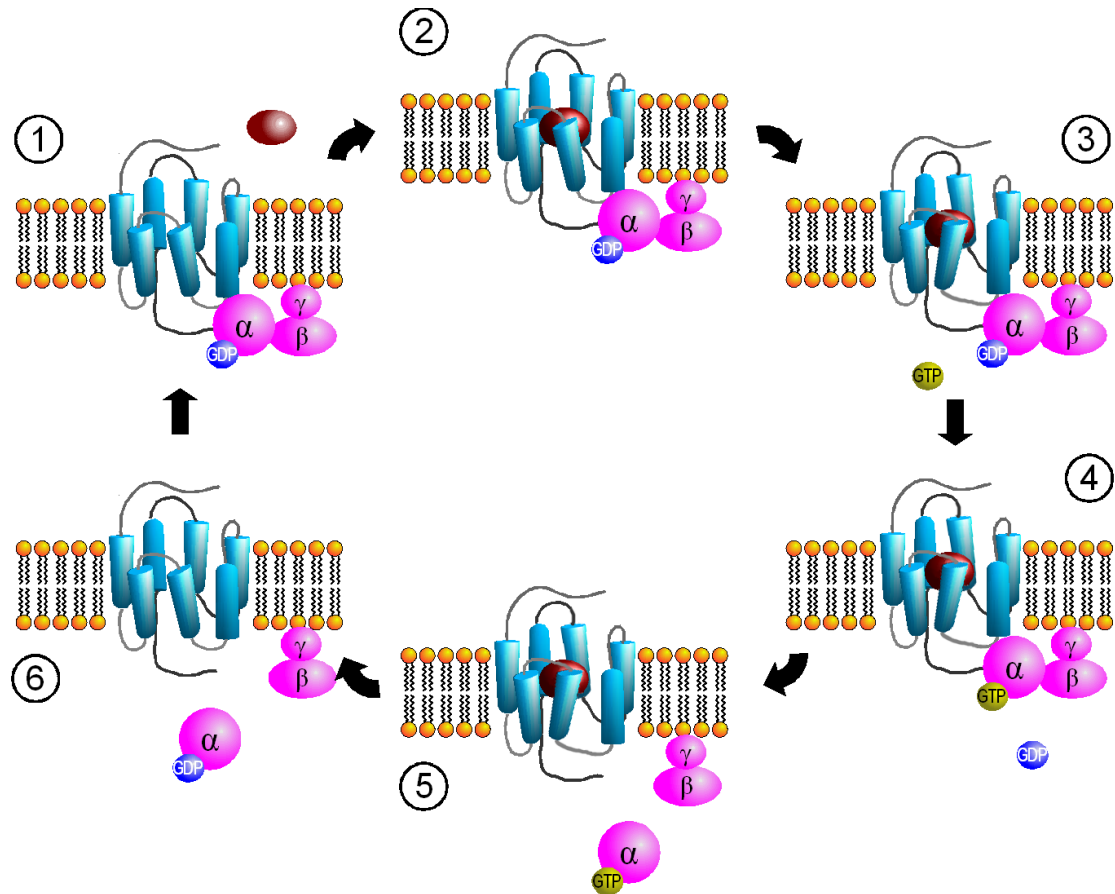


Figure 2: Activation cycle of G-proteins by G-protein-coupled receptors. 1-An agonist binds to the G-coupled protein receptor. 2- Agonist activates the G-coupled protein receptor. 3- Conformational change takes place. 4- Due to the conformational changes the attachment of GTP to the α -subunit takes place 5- α -subunit with GTP dissociates from the β - γ stable dimer. Both the α -subunit and β - γ stable dimer modulate several cellular signaling pathways. 6- Once energy is used the α -subunit with GDP reunites with the β - γ stable dimer until it is activated again. Adapted from Jähnichen S, 2006.

OR have also been shown to promote Ca^{2+} influx, stimulate phosphoinositide hydrolysis and stimulate adenylyl cyclase (Powell *et al.*, 2002). Figure 3 illustrates one example of the mechanism of opioid action.

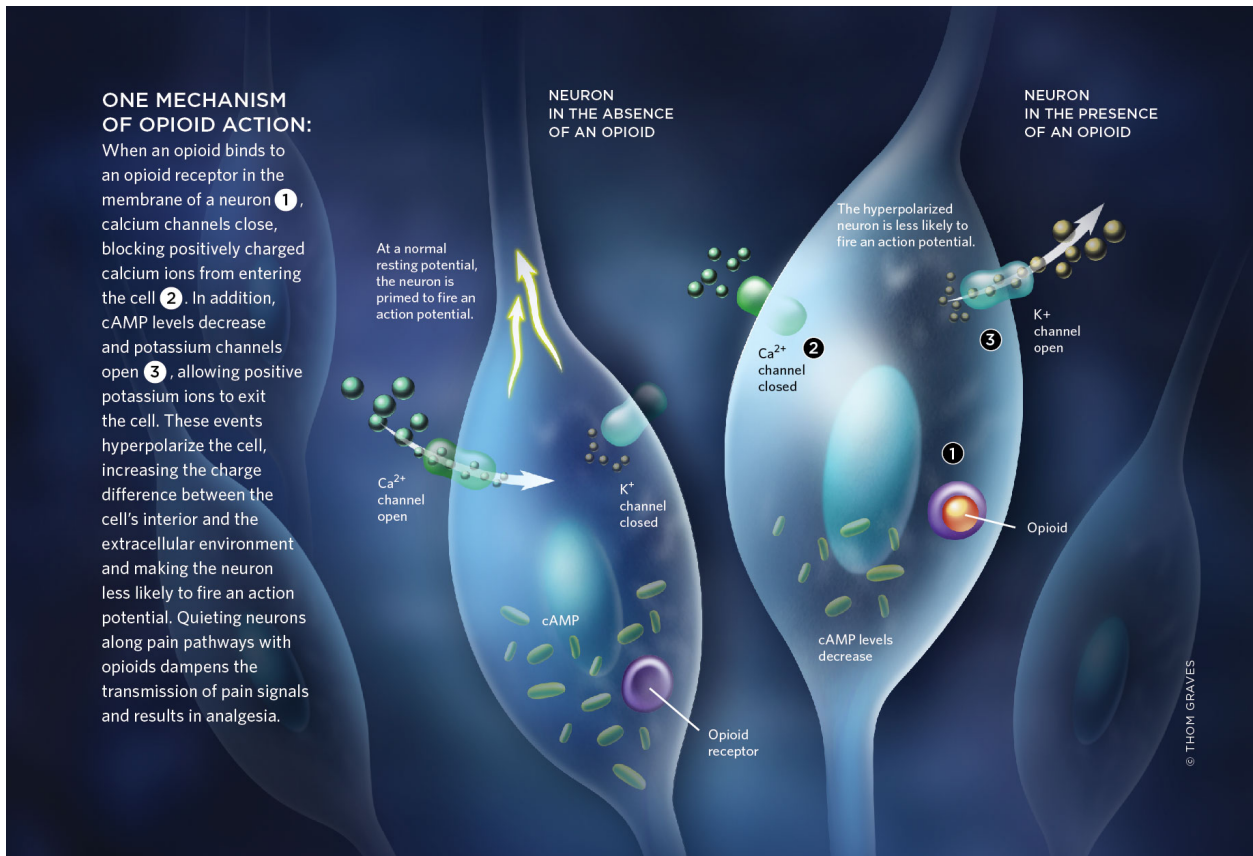


Figure 3: One example of the mechanism of opioid action. When an opioid binds to an OR in the membrane of a neuron, calcium channels close, blocking positively charged calcium ions from entering the cell. In addition, cAMP levels decrease and potassium channels open, allowing positive potassium ions to exit the cell. These events hyperpolarize the cell, increasing the charge difference between the cell's interior and the extracellular environment and making the neuron less likely to fire an action potential. Adapted from Grens K, 2011.

The activation of all three types of OR by an agonist leads to the inhibition of adenylyl cyclase and modulation of membrane Ca²⁺ and K⁺ conductance. The increase in the K⁺ conductance and decrease in Ca²⁺ conductance by opioids reduces membrane excitability and contributes to the analgesic property of opioids. The inhibition of cAMP by opioids implies a more complex pathway for opioid regulation of cellular mechanism (Gong *et al.*, 1998).

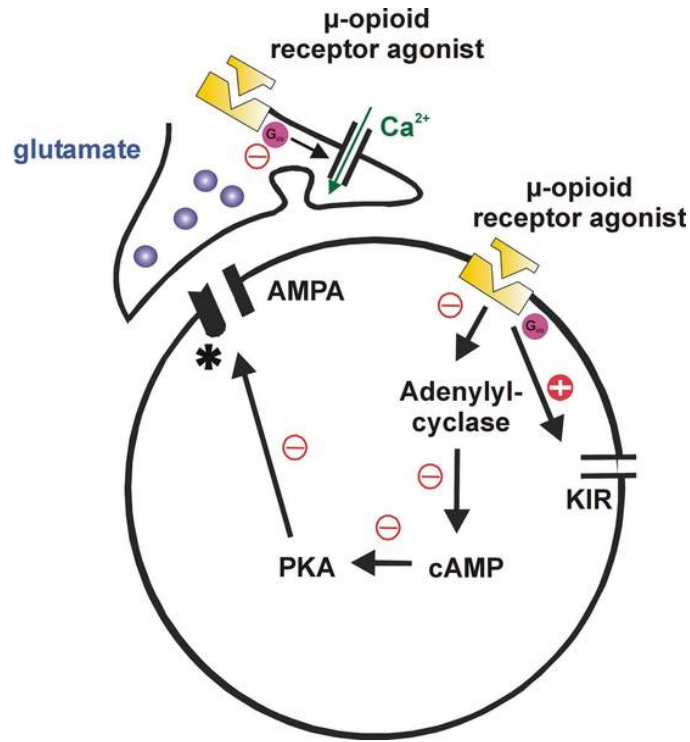


Figure 4: Pre- and post-synaptic effects of MOR agonists on synaptic transmission. Opioids specifically depress neurotransmitter release from nociceptive A δ -fiber and C-fiber via presynaptic inhibition mediated by an inhibition of N-type, and to a lesser extent of P/Q-type voltage-dependent Ca²⁺-channels. Opioids regulate neuronal excitability and transduce receptor activation to downstream signal transduction pathways via the postsynaptic inhibition.

Some opioids bind presynaptically to MOR on the central terminals of primary afferent nociceptive nerve fibres and others post synaptically on superficial dorsal horn neurons (Fig.4). KORs are located presynaptically on primary afferent neurons in the dorsal horn of the spinal cord where they participate in the inhibition of the release of excitatory neurotransmitter such as substance P, calcitonin gene-related peptide and glutamate. DORs are present on postsynaptic terminals of secondary order neurons and they decrease the excitability caused by the activation of other postsynaptic receptors such as neurokinin-1 receptor and N-methyl-D-aspartate. MORs are located either at presynaptic or postsynaptic terminals, therefore, they can either modulate the release of excitatory neurotransmitters or decrease the excitability of postsynaptic receptors (McDonald & Lambert, 2015). Presynaptic MORs not only inhibit neurotransmitter release of gamma-amino butyric acid (GABA), but they also decrease the inhibition of dopamine pathways causing more dopamine to be released. Exogenous opioids through this mechanism cause inappropriate dopamine release which leads to abnormal synaptic plasticity causing addiction.

I.3.1 Endogenous opioids

Endogenous opioids are opioid peptides released by neurons. There are four families of endogenous opioid peptides: endorphins, enkephalins, dynorphins and a recently classified family called endomorphins. All these peptides are derived by the cleavage of larger protein precursors. The precursors prepro-opiomelanocortin, preproenkephalin and prodynorphin, which are encoded by three corresponding genes code for enkephalins, endorphins and dynorphins respectively (Koneru *et al.* 2009).

I.3.1.1 Endorphins

Endorphins are endogenous polypeptides that are considered natural painkillers. In vertebrate animals during strenuous exercise, excitement, pain and orgasms, the hypothalamus and pituitary gland produces endorphins, which causes the feeling of well-being and analgesia (Sprouse-Blum *et al.*, 2010). Endorphins act by binding to MOR, hence causing analgesia and a sense of euphoria. They also cause the release of many sex hormones. There are four types of endorphins present in the body α -, β -, δ - and γ -endorphins, the most important and powerful endogenous opioid neurotransmitter is the β -endorphins. In the CNS, β -endorphins primarily bind to the presynaptic MOR inhibiting the release of GABA, thus resulting in excess production of dopamine. In the peripheral nervous system, β -endorphins bind to MOR both pre- and post-synaptically and inhibit the release of tachykinin, particularly substance P, a key protein responsible for the transmission of pain (Sprouse-Blum *et al.*, 2010). β -endorphins have the highest affinity for MOR, with medium affinity for DOR and lowest affinity to KOR (Holzer, 2014).

I.3.1.2 Enkephalins

Nociception in the body is partly regulated by pentapeptides called enkephalins. Proenkephalin and prodynorphin are proteolytically cleaved into enkephalin peptides (Orduna & Beaudry, 2016). Enkephalins exist mainly in two forms: met-enkephalin and leu-enkephalin, both of which are found in abundance in the brain and endocrine tissues (Michael Comb *et al.*, 1982). They are also expressed in the gut, where OR agonists interact with pathways of the enteric nervous system that regulate GI motility and secretion (Hughes *et al.*, 1977). They play an

important role in the behavior, cardiac function, cellular growth, immunity and ischemic tolerance. Among their functions are pain perceptions, mood and behavior by altering emotional responses as well as acting on cardiovascular and respiratory functions (Przewlocki & Przewlocka, 2001). Enkephalins are distinguished by their C-terminal amino acid sequence, the opioid motif of met-enkephalin consists of Tyr–Gly–Gly–Phe–Met, whereas for leu-enkephalin it consists of Tyr–Gly–Gly–Phe–Leu (M. Comb *et al.*, 1982). Their selectivity for DOR is moderately higher than MOR (Nieto *et al.*, 2005).

I.3.1.3 Dynorphins

Dynorphins are formed by the cleavage of precursor proteins called prodynorphin by protein convertases (Berman *et al.*, 2000; Day *et al.*, 1998). Two types of dynorphins are formed: dynorphin A and dynorphin B. They are a family of endogenous opioid peptides that have potent analgesic effects and have been identified as neuropeptides involved in endogenous pain inhibition (Kuner, 2010; Mika *et al.*, 2011). They are produced in different parts of the brain including midbrain, hippocampus, pons, medulla and spinal cord. They are also responsible for homeostasis, appetite control, pain regulation and body temperature regulation (Koneru *et al.* 2009). They exert their effects through KOR. The KOR and the endogenous dynorphins are rich in the ventral tegmental area, nucleus accumbens, and prefrontal cortex, brain regions that regulate mood and motivation. Neurochemical and electrophysiological data have shown that KOR activation in these regions decreases dopamine transmission (Shippenberg *et al.*, 2007) that maybe effective in the treatment of depression and drug addiction (Shippenberg, 2009).

I.3.1.4 Endomorphins

Endomorphins have a key role in pain perception, responses related to stress and complex functions such as reward, arousal and vigilance (Fichna *et al.*, 2007). These tetrapeptides differ from the other known endogenous opioid peptides through their amino acid sequence, in which tyrosine residue is followed by glycine. All other endogenous opioids share the Tyr-Gly-Gly-Phe amino acid sequence at the N-terminus. Endomorphins are widely distributed in the CNS and have the highest known affinity and specificity for MOR. There are two types of endomorphins: endomorphin1 and endomorphin2. Both inhibit nociceptive transmission in the spinal cord through MOR (Przewlocki *et al.*, 1999).

Endogenous opioids	Affinity for OR		
	MOR	DOR	KOR
β -Endorphins	+++	++	-
Endomorphin-1	+++	-	-
Endomorphin-2	+++	-	-
Met-enkephalin	++	+++	-
Leu-enkephalin	+	+++	-
Dynorphin-A	+	-	+++
Dynorphin-B	+	+	+++

Table 1. Summary of the affinity of endogenous opioids to specific receptors. (Merg *et al.*, 2006; Raynor *et al.*, 1994; Stein *et al.*, 2009)

OR	CNS location	Response on activation
MOR	Brain (laminae III and IV of the cortex, thalamus, periaqueductal gray), spinal cord (substantia gelatinosa)	supraspinal analgesia, physical dependence respiratory depression, miosis, euphoria, reduced GIT motility
DOR	Brain (pontine nucleus, amygdala, olfactory bulbs, deep cortex).	Analgesia may be associated with mood change
KOR	Brain (hypothalamus, periaqueductal gray, claustrum), spinal cord (substantia gelatinosa)	Spinal analgesia, diuresis, dysphoria, sedation, miosis, disrealization, depersonalization

Table 2. Summary of OR, their tissue location and the response to their activation resulting from the binding of endogenous opioids onto the receptors. Adapted from (Cherny, 1996). OR are also present in the autonomic nervous system, peripheral nerves and GIT, where they can mediate effects on heart rate, nociception and GIT motility.

I.4 Pharmacological drugs targeting opioids receptors

I.4.1 Morphine

Morphine is a naturally occurring opiate found in opium plant, *Papaver somniferum*. It acts on the MOR present in the CNS to relieve pain. It is used to treat moderate to severe pain. Morphine is generally prescribed for inpatients or post-operative patients, it is also given to terminally ill patients. It has high abuse potential. Morphine exerts its effect by binding to MOR, KOR and DOR, but morphine has a higher affinity for MOR. Morphine can be administered orally, intravenously, rectally, subcutaneously, epidurally, by inhalation and by snorting (Martin *et al.*, 2016). The liver metabolizes morphine to morphine-6-glucuronide (M-6-G) and morphine-3-glucuronide (M-3-G) both of which are then excreted through the kidneys. M-6-G is more potent than morphine (Frances *et al.*, 1990) and it is said to have higher affinity for the DOR than the MOR. M-6-G is also suggested to be safer than morphine because it causes lower respiratory depression due to the difference in their affinities towards the OR (Kilpatrick & Smith, 2005). The side effects of morphine include nausea, vomiting, constipation, myosis, sedation, drowsiness, allergy, euphoria, delirium, tolerance and respiratory depression. The adverse effects of morphine on the recovery of locomotor function may be linked to the activation of the KOR (Aceves *et al.*, 2016). Studies performed on knockout mice lacking the MOR, show that mice display no morphine-induced analgesia (Matthes *et al.*, 1996) or respiratory depression (Romberg *et al.*, 2003) with morphine, suggesting that the two effects are closely linked. Morphine is the standard drug against which all other opioid analgesics are compared.

I.4.2 Codeine

Codeine has significantly lower affinity to MOR in comparison to morphine (Knaggs *et al.*, 2004). Codeine is converted to morphine by CYP2D6 in the liver and imparts its analgesic action through morphine (Desmeules *et al.*, 1991; Sindrup *et al.*, 1990). It is also converted by CYP3A4 to norcodeine, which is reported to be an inactive metabolite. It is considered to be the agent of choice for the treatment of mild to moderate pain in both adults and children. Side effects of codeine include dizziness, shortness of breath, nausea and vomiting, which are all due to its

opioid effect. Severe adverse reactions to codeine include respiratory depression, circulatory depression, respiratory arrest, shock and cardiac arrest (Barh *et al.*, 2013).

Codeine is used as an antitussive in some cough-suppressing drugs. Cough is normally caused by the activation of cough reflex arc that includes vagal afferent nerves, cough center in the medulla and the efferent nerves. Inhibition at any site of this arc relieves cough. The antitussive effect of codeine is thought to be primarily by activation of the MOR and KOR receptors. Binding studies have demonstrated that codeine is more selective for MOR than KOR or DOR receptor (Kotzer *et al.*, 2000; Mignat *et al.*, 1995). KOR agonists also have antitussive activity, therefore, both MOR and KOR are considered as candidates for being the receptors, which contribute to its antitussive activity (Takahama & Shirasaki, 2007). Codeine is one of the well-characterized drugs used for analyzing the function of CYP2D enzyme in the liver. An elaboration on codeine, its pharmacokinetics, pharmacogenomics and pharmacodynamics is discussed in chapter III.

I.4.3 Oxycodone

Oxycodone is a semisynthetic opioid analgesic made from thebaine, a naturally occurring opioid alkaloid in the opium plant (Freund *et al.*, 1999). Oxycodone is prescribed for moderate to severe pain and produces analgesia via the MOR. Oxycodone also has some affinity for a subtype of KOR and little to no affinity for DOR (Lalovic *et al.*, 2006; Nielsen *et al.*, 2007; Virk & Williams, 2008). *In vitro* and *in vivo* data suggest differences in the mechanism of action of morphine and oxycodone (Smith, 2008). Pretreatment with norbinaltorphimine, a KOR-selective antagonist blocks antinociception of intracerebroventricular oxycodone, but not morphine and pretreatment with MOR-selective antagonist naloxonazine blocks intracerebroventricular morphine but not oxycodone (Nielsen *et al.*, 2007).

I.4.4 Naloxone

Naloxone is known to block or reverse the effects of opiate medications, which includes extreme drowsiness, respiratory depression, mental depression and loss of consciousness (Hardman JG & Limbird LE, 2006), and as such, is used for the treatment of mild to severe overdose with opioids. Reversal of respiratory depression caused by overdose with partial opiate agonists, may be incomplete or may require higher doses of naloxone. Intravenous naloxone acts within two

minutes and its effect lasts for thirty minutes, because of its short time effect compared to longer acting effect of opioids, multiple doses of naloxone is required to reverse the effects of opioids. In opioid abusers, naloxone can precipitate abrupt withdrawal symptoms such as body aches, restlessness, anxiety, tachycardia and seizure. Naloxone has been reported to be a MOR inverse agonist (Sirohi *et al.*, 2009). Published data suggests that naloxone has 9-fold higher affinity for MOR in comparison to KOR and 60-fold greater affinity to DOR (Codd *et al.*, 1995). Naloxone in the absence of opioids has not been documented to have any effect on the body since it is a competitive antagonist of OR.

I.4.5 Naltrexone

Naltrexone and its active metabolite 6 β -naltrexol are antagonists at the MOR, KOR to a lesser extent and DOR to a possibly insignificant extent. It is used in the management of opioid dependence. Despite being approved by the FDA for opioid addiction it is more frequently used for alcoholism. Ultra-low doses of naltrexone influence morphine analgesia and tolerance through spinal cord action. These doses increase acute morphine analgesia, whereas a high dose of naltrexone blocks analgesia. Furthermore ultra-low doses of naltrexone inhibits the development of morphine tolerance and partially restores morphine potency in animals previously showing tolerance (Ling *et al.*, 1986). Recent studies in mice showed that ultra-low doses of systemic naltrexone augment morphine-induced analgesia and inhibit the development of tolerance and/or physical dependence.

I.4.6 Tramadol

Tramadol is a centrally acting opioid analgesic closely related to codeine and morphine. Tramadol consists of two enantiomers, tramadol and O-desmethyltramadol, both of which contribute to analgesia via different mechanisms and both are agonists at MOR. Tramadol also inhibits serotonin and norepinephrine reuptake thus enhancing the inhibitory effects on pain transmission in the spinal cord. Tramadol is metabolized in the liver by CYP2D6 to O-desmethyltramadol, which is an active metabolite. It also gets metabolized by CYP2B6 and CYP3A4 to N-desmethyltramadol, an inactive metabolite. It undergoes Phase II conjugation reaction to form glucuronides and sulphates. Tramadol and its metabolites are mainly excreted by the kidneys. A wide variability in the pharmacokinetic properties of tramadol can be partially attributed to CYP polymorphism. Tramadol is effective in reducing pain caused by trauma, renal

or biliary colic, labour and chronic pain of malignant or non-malignant origin especially neuropathic pain. Due to its dual mechanism of action tramadol is useful in opioid-resistant chronic pain.

I.4.7 Fentanyl

It is a semisynthetic opioid that is 50 to 100 times more potent than morphine. It was introduced into clinical practise in the early 1960s. Fentanyl is a MOR agonist. It is used as an adjunct to general anaesthesia and to induce and maintain anaesthesia. The off label indications of fentanyl include its use as an analgesic for severe pain. Fentanyl contains a boxed warning for respiratory depression even at low therapeutic doses, which is caused by its direct action on the brain stem respiratory centers. It also depresses cough reflex by direct action on the cough center in the medulla. It is metabolized in the liver and intestinal mucosa by CYP3A4 isoform to norfentanyl. A study stated that fentanyl could modify the response to noxious pressure in inflamed paws via a peripheral site of action in rats, thus suggesting that it has possible role in the modulation of nociception in inflamed tissues via peripherally located opioid receptors (Stein *et al.*, 1988).

Drugs	Affinity for OR			Metabolic pathway in human
	MOR	DOR	KOR	
Morphine	Agonist	Weak agonist	Weak agonist	Glucuronidation
Codeine	Weak agonist	Weak agonist	-	CYP3A4, CYP2D6
Fentanyl	Agonist	Agonist	Weak Agonist	CYP3A4
Oxycodone	Agonist	-	Strong agonist	CYP3A4, CYP2D6
Oxymorphone	Agonist	-	-	Glucuronidation
Naltrexone	Antagonist	-	-	-
Naloxone	Antagonist	Antagonist	Antagonist	-
Tramadol	Agonist	-	-	-
Fentanyl	Agonist	-	-	-
Methadone	Agonist	Strong agonist	Weak agonist	CYP3A4, CYP2B6, CYP2C8, CYP2C19

Table 3: Affinity of some of the commonly used opioids along with their metabolic pathway and the effect produced (Smith & Peppin, 2014).

I.5 Codeine

I.5.1 Physico-chemical properties of codeine

Codeine is chemically known as methyl morphine ($C_{18}H_{21}NO_3$) and has a molecular weight of 299.36424 g/mol. It is obtained either naturally from *Papaver somniferum* plant or by methylation of morphine.

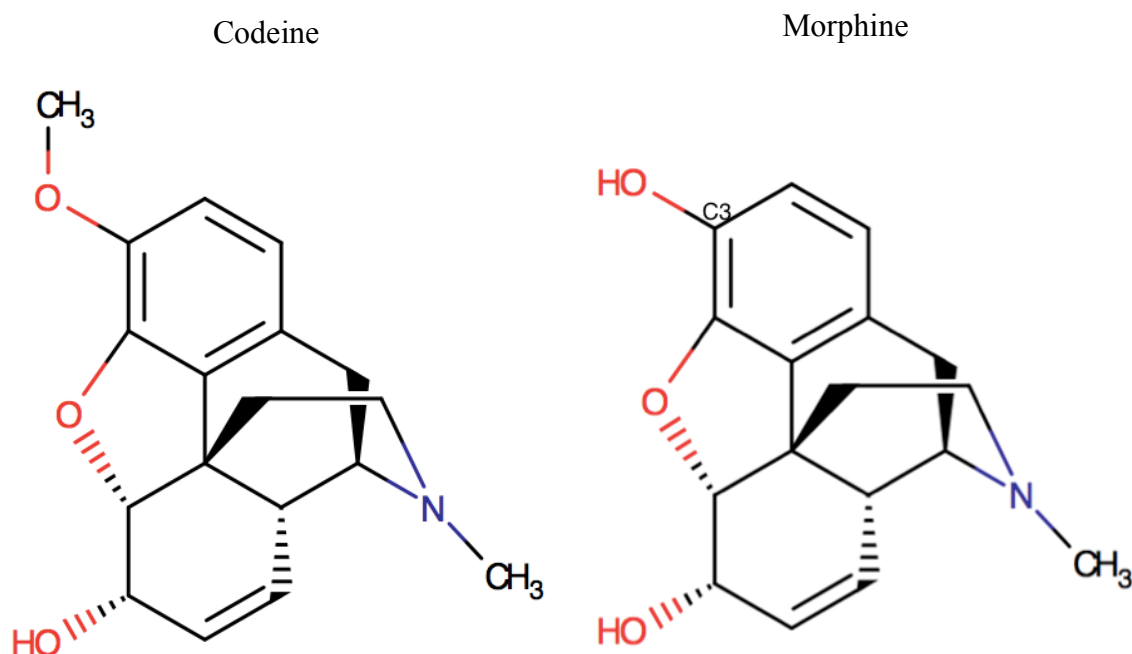


Figure 5: The molecular structures of codeine and morphine.

The stronger analgesic activity of morphine is attributed to the free hydroxyl group at C3 enabling a high affinity receptor binding whereas a low affinity receptor binding was reported for codeine (Mignat *et al.*, 1995). The methylation of the hydroxyl group of morphine led to a more lipophilic compound that is codeine (Kay *et al.*, 1967).

I.5.2 Metabolism of codeine

Codeine is metabolized in the liver by CYP2D and CYP3A to form primary metabolites including morphine and norcodeine (Caraco *et al.*, 1996). Figure 6 represents the metabolic pathway of codeine in the liver. Codeine by itself is not an analgesic; it is only through its

conversion to morphine by CYP2D in the liver that it expresses its analgesic effect (Desmeules *et al.*, 1991; Sindrup *et al.*, 1990).

The affinity of codeine to the MOR is very low compared to morphine, which has 200 folds higher affinity for the receptor than codeine (Chen *et al.*, 1991; P. Madadi & Koren, 2008; Yue *et al.*, 1991). Morphine is one of the most potent opioid drugs present in the market and hence the analgesic effect felt after taking codeine is, without doubt, due to the morphine generated after its metabolism. Morphine is further converted to M-6-G and M-3-G by glucuronidation. Since codeine is metabolized by CYP2D enzyme into morphine, any changes in the enzymatic activity of CYP2D will lead to a variation in its pharmacological effect, which includes codeine efficacy in terms of pain relief, toxicity due to its accumulation and side effects exacerbation.

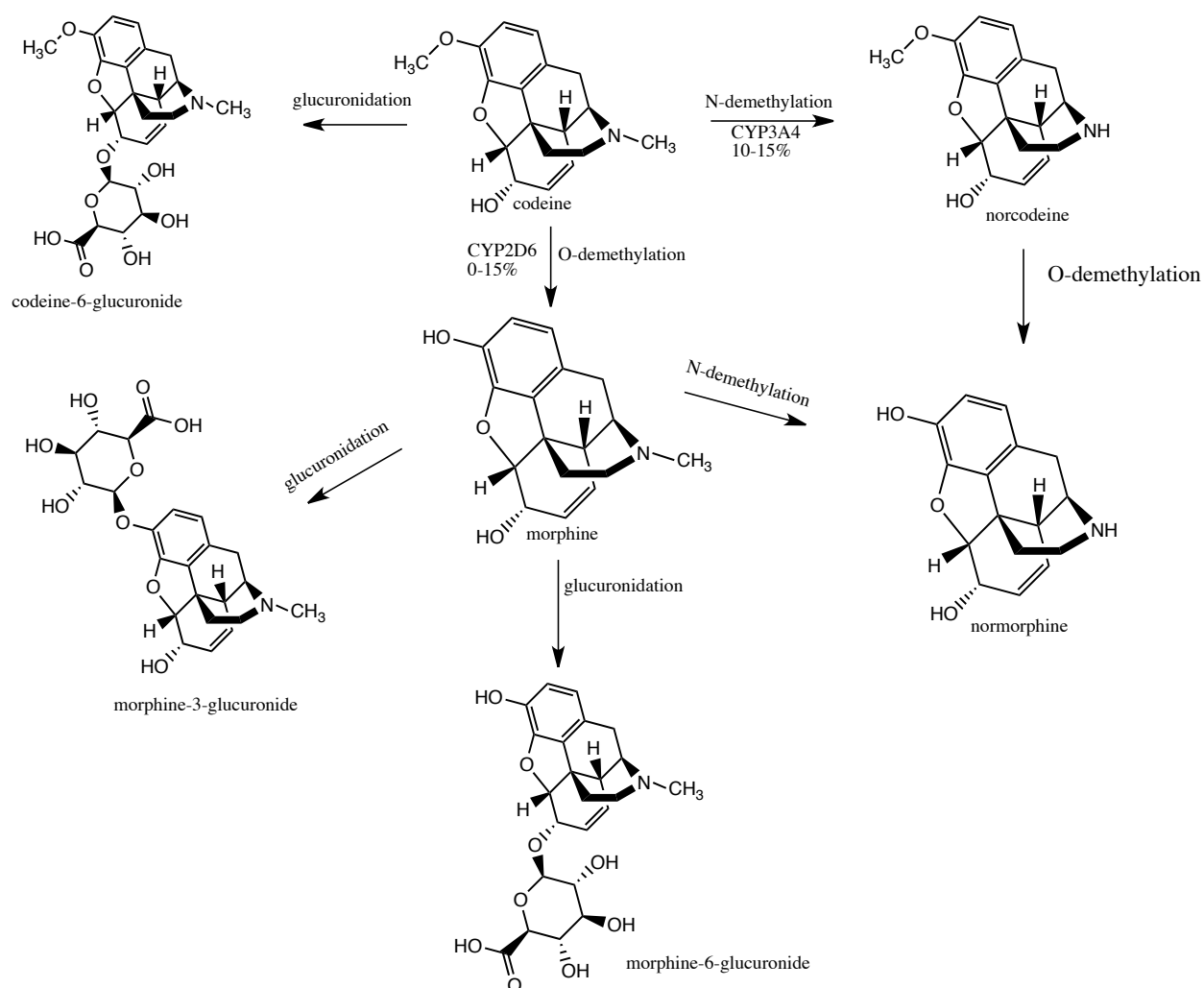


Figure 6: Drug-metabolizing enzymes involved in the metabolism of codeine. In human, codeine is bioactivated by CYP2D6 to morphine and demethylated by CYP3A4 to norcodeine an inactive metabolite. Adapted from Stamer *et al.* 2010.

I.5.3 Effects of codeine:

Codeine acts on the CNS by acting as an analgesic. Other effects include anxiolysis, euphoria and feelings of relaxation. A direct effect of codeine on the brain stem respiratory centres can cause respiratory depression. On the vascular system, it is known to produce peripheral vasodilatation that could cause orthostatic hypotension and fainting. Similar to other opioids, it can cause histamine release, which plays a role in opioid-induced hypotension. Histamine release may cause pruritus, flushing, red eyes and sweating. Effects of codeine on the GIT include decrease in gastric, biliary and pancreatic secretions resulting in indigestion. It can also cause a reduction in motility and an increase in tone in the atrum of the stomach and duodenum. In the small intestine, propulsive peristaltic waves in the colon are decreased, while tone is increased which results in constipation. Table 4 is a summary of codeine and some of its effects on important organs in the body.

Codeine	
Site	Effects
Central nervous system	Analgesia, anxiolysis, euphoria, relaxation.
Respiratory system	Respiratory depression.
Cardiovascular system	Orthostatic hypotension
Gastrointestinal tract	Decrease gastric, biliary and pancreatic secretions; decrease gastrointestinal tract motility, constipation.

Table 4: Codeine and some of its effects on important organs in the body.

I.5.4 Excretion

Codeine and its metabolites are excreted primarily by kidneys in humans, monkeys and dogs (Casarett & Doull, 1977). Urinary excretion is rapid with approximately 67% of total alkaloids being eliminated in the urine within 6 hours after the administration of the drug. Excretion is almost complete by 24 hours, but trace amounts of codeine and its metabolites can still be found in urine for several days. Codeine metabolites were determined by using HPLC in urine of male rats. Unchanged codeine, codeine glucuronide, free morphine and morphine-3-glucuronide were detected in 24 hours urines of the rats. Codeine is metabolized by glucuronidation and by oxidative N- and O-demethylation in rats (Oguri *et al.*, 1990)

I.5.5 Pharmacokinetics

Codeine is readily absorbed from the GIT and has a reported oral bioavailability of $80.5\% \pm 30.9\%$ (Guay *et al.*, 1988). It is rapidly distributed from blood to body tissues, passes the blood brain barrier and is found in fetal tissues and breast milk. Lipophilicity of codeine from reported literature was found to be 0.60 (octanol/pH 7.4) + 0.89 (Moffat *et al.*, 2005; Moffat, 1986). The apparent volume of distribution of codeine has been reported to be approximately 3-6 L/kg indicating extensive distribution of the drug into tissues. The total body clearance, renal clearance and non renal clearance of codeine were reported to be 15.0 ± 3.4 ml/min/kg, 1.71 ± 0.78 ml/min/kg and 13.32 ± 2.83 ml/min/kg (Guay *et al.*, 1988). The reported half-life of codeine in humans is about 2.9 hours.

Table 5 presents data collected from different publications where the pharmacokinetics of codeine was evaluated.

Species	Dose	AUC	V _d	CL	T _{1/2}	Reference
Rats	3mg/kg (i.v)	30279 ng.min/mL	5.1 L/kg	6.3 l/kg/hr	39.6 min	(Shah & Mason, 1990)
Rats	5mg/kg (oral)	4074 ng.min/mL	-	6.2 l/kg/hr	34.1 min	
Greyhound dogs	1.43 mg/kg (oral)	30.53 h.ng/mL	-	-	1.6 h	(KuKanich, 2010)
Beagles dogs	0.9 mg codeine/ml in solution (oral)	8.34 ng/h/mL	-	-	2.67 h	
Humans						
PM	30 mg	180 ug.h/l	-	-	4.8 h	(Kirchheiner <i>et al.</i> , 2007)
EM	30 mg	191 ug.h/l	-	-	3.6 h	
UM	30 mg	192 ug.h/l	-	-	3.7 h	
Humans	60mg/70kg	734 h.ug/l	-	-	2.1 h	(Kim <i>et al.</i> , 2002)
	120mg/70kg	1800 h.ug/l	-	-	2.4 h	
Humans	60 mg (oral)	1262.4 ng.mL/h	-	-	2.6 h	(Band, Band, Deschamps, Besner, & Coldman, 1994)
Humans	30 mg (oral)	7.37%	389 l	2280 ml/min	1.47 h	(Vree & Verwey-van Wissen, 1992)
Humans						
EM	50 mg (oral)	1010 nmoll ⁻¹ h		2.10 l/h/kg	2.43 h	(Yue <i>et al.</i> , 1991)
PM	50 mg (oral)	1020 nmoll ⁻¹ h		1.92 l/h/kg	2.90 h	
Humans	60 mg (oral)	649 ng/ml/hr	6 L/kg	15.3 ml/min/kg	4.5 h	(Guay <i>et al.</i> , 1988)

Table 5: Pharmacokinetic data of codeine collected from different publications show a wide variability in the values reported. The – represents values that were not reported. Abbreviations PM, EM and UM described in legend.

I.5.6 Pharmacogenetics

Recent studies showed that the pharmacokinetics of codeine varies between individuals and can only be described via pharmacogenomics of CYP2D6 polymorphism. Humans can be categorized according to CYP2D6 polymorphism into poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultra-rapid metabolizers (UM). CYP2D6 alleles are characterized as normal function, reduced function and non-functional based on the expected activity level of the enzyme for which they encode (Crews *et al.*, 2012). Two non-functional alleles result in PM phenotype, at least one reduced functional allele in IM and at least one functional allele in EM, multi copies of a functional allele, due to duplication or multiple duplications of the CYP2D6 gene in UM phenotype (Table 4). In UM and EM a large dose of codeine is metabolized to morphine leading to morphine toxicity that can cause severe respiratory depression and death. In PM codeine is not converted to morphine and hence the affected individuals do not experience any analgesia from codeine and thus continue to experience pain (Table 6).

Likely phenotype	Genotypes	Implications of codeine metabolism	Codeine therapy recommendation
Ultrarapid metabolizer	An individual carrying more than two copies of alleles.	Increased formation of morphine following codeine administration leading to higher risk of toxicity.	Avoid codeine use due to potential toxicity.
Extensive metabolizer	An individual carrying two alleles encoding full function allele together with reduced-function allele.	Normal morphine formation.	Use label recommended age or weight-specific dosing.
Intermediate metabolizer	An individual carrying one reduced and one non-functional allele.	Reduced morphine formation.	Use label recommended age or weight-specific dosing. If no response, consider alternative analgesics such as morphine or non-opioid.
Poor metabolizer	An individual carrying no functional alleles.	Greatly reduced morphine formation following codeine administration leading to insufficient pain relief.	Avoid codeine use due to lack of efficacy.

Table 6: Recommended dosage of codeine based on CYP2D6 phenotypes. Adapted from Crews *et al.*, 2012.

Studies show that morphine AUC after codeine administration varied more than 30-fold between PM and UM groups (Johansson *et al.*, 1991; Mikus *et al.*, 1994; Mortimer *et al.*, 1990; Yue, *et al.*, 1997). A strong correlation between the number of active CYP2D6 genes and plasma concentrations as well as urinary recovery ratios of all O-demethylated codeine metabolites was observed. The plasma concentrations and AUCs of morphine between UMs and EMs differed about 1.5-fold with a nearly exact linear gene-dose effect. There was an almost exact linear relationship between the number of active CYP2D6 gene copies and codeine total clearance. The metabolic ratios calculated as codeine/morphine and (codeine+Codeine-6-Glucuronide)/(morphine+M-3-G+M-6-G) varied significantly depending on CYP2D6 activity. Cases have been reported of adults with UM phenotype suffering opioid toxicity even with small doses of codeine (Madadi *et al.*, 2009).

In PMs, the ratios were about 10-fold higher compared to EM or UM. A significant trend towards lower ratios with increasing CYP2D6 activity was observed. UM resulted in a 1.5-fold higher morphine production compared to EM. This difference is only moderate but the risk for opioid intoxication might be increased in UMs if other additional factors such as reduction in renal function or inhibition of other enzyme systems occur at the same time (Kirchheiner *et al.*, 2007). When sedation and miosis as opioid effect-related parameters are observed a slight decrease in pupil diameter in all groups was observed but no CYP2D6 genotype-related effects on miosis have been detected. A longer period of miosis was observed in PM though not statistically significant (Kirchheiner *et al.*, 2007). Significant variability in both the pharmacokinetics and pharmacodynamics of codeine has been shown in animal and adult human laboratory experimental studies (Chen *et al.*, 1991; Cleary *et al.*, 1994; Eckhardt *et al.*, 1998; Mikus *et al.*, 1991).

I.5.7 Effect of age

It has been suggested that neonates and infants have a reduced metabolic capacity for codeine as CYP2D6 activity is, at most, less than 1% of the adult values in foetal liver microsomes (Quiding *et al.*, 1992). CYP2D6 expression rapidly reaches adult levels within the first 6 months of age (Stevens *et al.*, 2008). Although another study suggests that enzyme activity may be less than 25% of adult values up to 5 years of age (Tateishi *et al.*, 1997). CYP2D6 genotype seems to

be concordant at 2 weeks of age (Madadi *et al.*, 2009). A significant relationship between phenotype and plasma morphine was shown in children (Sindrup & Brøsen, 1995).

The United States Food and Drug Administration (FDA) have recently issued a boxed warning contraindicating the use of codeine after tonsillectomy and/or adenoidectomy in children. Post operatively, children suffering from obstructive sleep apnea passed away after therapeutic doses of codeine were prescribed. The children were later confirmed to be UM of codeine and may have suffered from breathing problems, but it is hard to determine UM in children and hence codeine has been contraindicated (Ciszkowski *et al.*, 2009; Kelly *et al.*, 2012). Three fatal or life threatening cases have been reported in children. The fatal cases had functional gene duplication encoding for CYP2D6 caused a greater production of potent morphine from codeine. Severe respiratory depression in an EM was also reported (Caraco *et al.*, 1996). Health Canada has also reviewed the safety of prescription of codeine in children and it no longer recommends its use in children less than twelve years of age. It can be used to stepdown from stronger opioids in instances when a patient's pain is decreasing over time (Ginsburg *et al.*, 2009).

In children antitussive dosages of 3-5 mg/kg/d have produced somnolence, ataxia, miosis, vomiting, rash, facial swelling and pruritus. Respiratory depression requiring mechanical ventilation occurred in 3% of children receiving doses greater than 5 mg/kg/d. Hepatic glucuronidation pathway is completely underdeveloped in infants, which places them at particular risk for adverse dose-related effects. In adults a linear relationship has been shown to exist between a codeine dosage in the range of 7.5-60 mg/d and a decrease in chronic cough (Crews *et al.*, 2012).

Available research findings imply that age specific differences in the pharmacokinetics of codeine may be significant. In a comparison of i.m and rectal codeine administration in children aged 3 months to 12 years, peak plasma levels were achieved between 30 and 60 minutes in both groups but rectal bioavailability was found to be lower. The plasma drug concentration data indicates that a rectal dose of codeine of 0.5 mg/kg in children can result in similar or slightly greater plasma concentration of codeine and its metabolites than after 60 mg in adults (Williams *et al.*, 2002).

Furthermore, nursing mothers are advised not to take codeine. In one case, a 13-day old breast fed infant was reported dead, but the mother was on a less than usual amount of codeine prescribed for episiotomy. She was genetically tested and confirmed to be an UM. The amount of morphine in her breast milk was at a toxic level and hence the baby died of opioid toxicity (Madadi *et al.*, 2007). Codeine is considered comparatively safe in the elderly and is still being prescribed (Chau *et al.*, 2008). A case of an elderly patient complaining of no pain relief after taking therapeutic doses of codeine, patient was confirmed to be a PM of codeine (Susce *et al.*, 2006).

The role of codeine in treatment of the elderly is a subject of debate due to lack of evidence and common adverse effects experienced by them. Assessment, evaluation and treatment of pain can be very challenging due to alterations in the pharmacokinetics of opiates, which occur with normal physiological aging, polypharmacy, multiple comorbidities and the potential of more side effects or treatment failures are other problems that should be taken in consideration. Codeine is considered a safe analgesic however there have been calls to withdraw it from the market. A meta-analysis of opioids found that the modest benefits of codeine were outweighed by adverse effects in the treatment of osteoarthritis of the knee or hip (Iedema J., 2011). A cohort study found that the risk of injury was higher in older people using combination products of codeine than those taking other sedative drugs (Buckeridge *et al.*, 2010).

The variation in pharmacological effect from children dying of morphine toxicity to elder patients experiencing no analgesic effect with increased risk of adverse effects, makes us assume that there is a change in CYP2D6 activity with age. The variation of CYP2D6 metabolic activity with age, may lead to a change in phenotype from UM and EM metabolizers to PM. The patients may hence experience no analgesia and codeine might accumulate in their body systems causing opioid toxicity and lack of pain relief. Overall the efficacy and side effects of codeine in different age groups has not so far been sufficiently investigated.

I.6 Oxycodone

I.6.1 Physico-chemical properties of oxycodone

Oxycodone is a semisynthetic opioid derived from the naturally occurring opium alkaloid, thebaine. Chemically oxycodone is a 4,5- α -epoxy-14-hydroxy-3-methoxy-17-methylmorphinon-6-onehydrochloride. The molecular formula of oxycodone is $C_{14}H_{21}NO_4$ and its molecular weight is 315.36364g/mol.

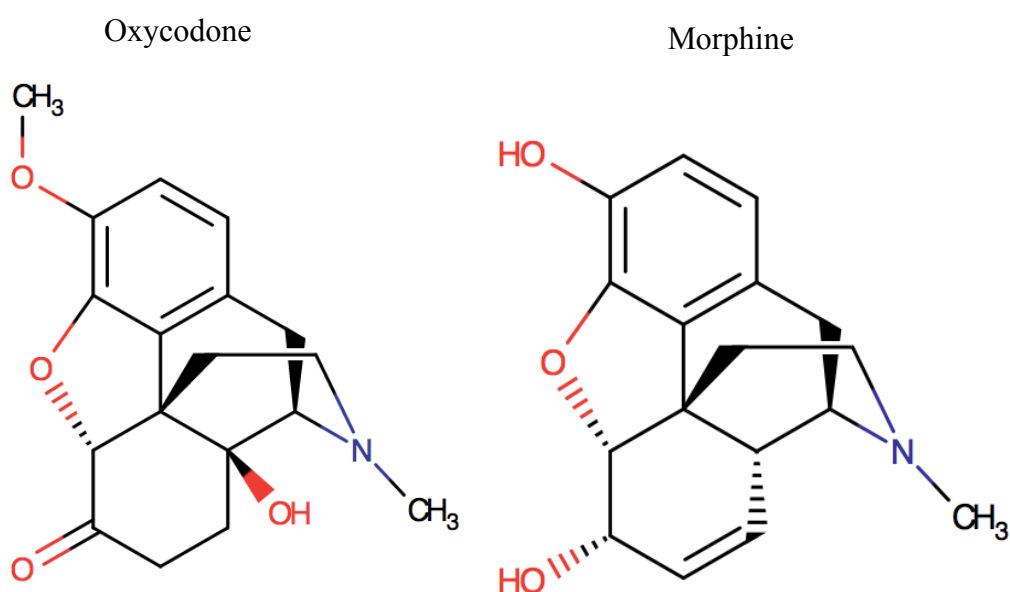


Figure 7: The chemical structures of oxycodone and morphine.

The structural difference between oxycodone and morphine means that oxycodone is not directly susceptible to phase II metabolism therefore it has greater bioavailability of 60-87% (Ordóñez *et al.*, 2007). Also because of the difference in structure, oxycodone causes less immunosuppression in comparison to other opioids.

I.6.2 Metabolism of oxycodone

In human, oxycodone is extensively metabolized by CYP2D6 and CYP3A4 in the liver to oxymorphone and noroxycodone respectively (Kalso, 2005). Systemic exposure to oxycodone and metabolically derived noroxycodone was approximately 2.3- and 6.4-fold higher in Dark

Agouti rats compared to Sprague-Dawley rats following a subcutaneous administration of single bolus of oxycodone at 2mg/kg whereas the circulating concentrations of metabolically derived oxymorphone remained very low (Huang *et al.*, 2005). Oxymorphone is a more potent opioid receptor agonist than oxycodone, whereas noroxycodone is less potent and has weaker antinociception than the parent drug (Kalso, 2005). Noroxycodone undergoes further oxidation by CYP3A4 to noroxymorphone, which is active at opioid receptors but it, does not seem to cross the blood brain barrier. The glucuronide metabolic pathway for oxycodone has so far not been established. The analgesic effect of oxycodone is due to the parent drug itself but some analgesia is a contribution of one of the active metabolites oxymorphone, which is more potent than oxycodone, but is formed in small quantities.

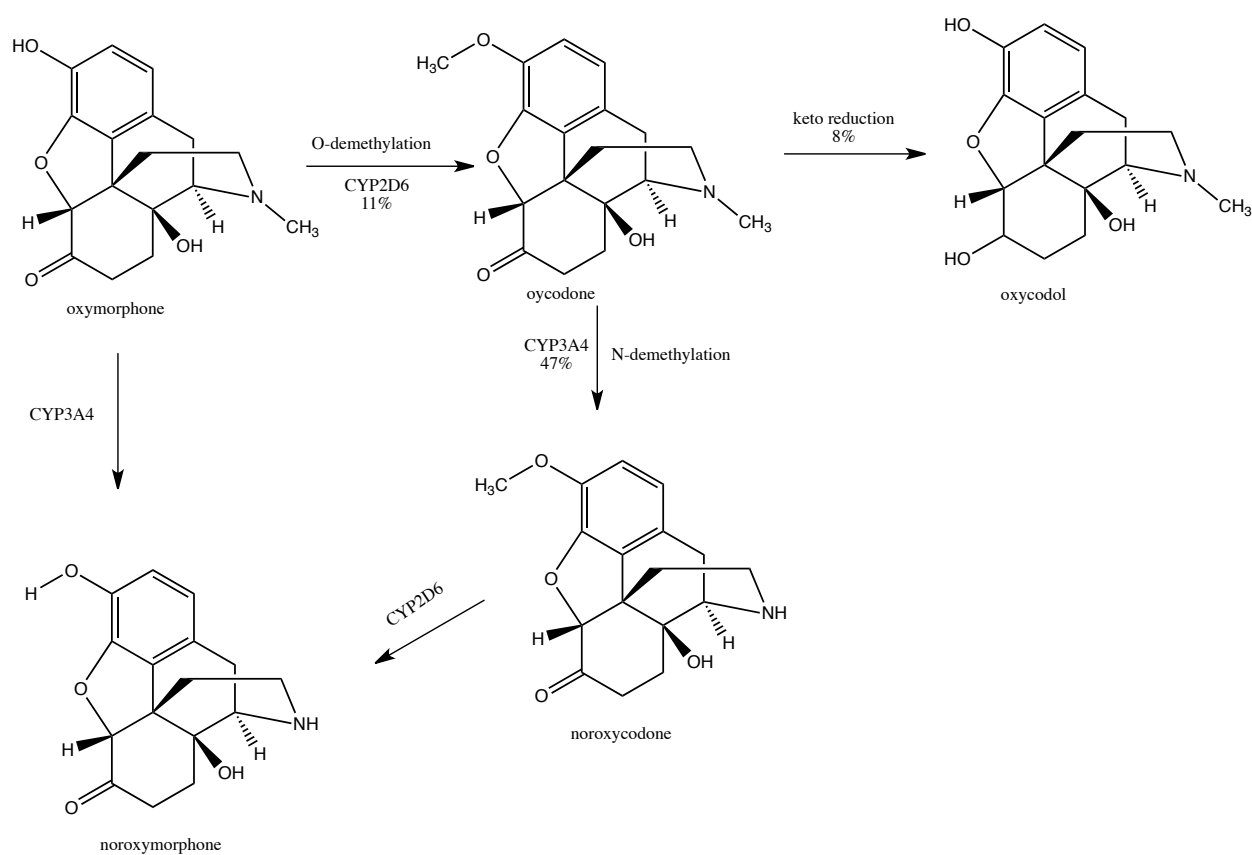


Figure 8: Drug-metabolizing enzymes involved in the metabolism of oxycodone. The major metabolic pathway of oxycodone is the formation of noroxycodone via CYP3A4 enzymes. Noroxycodone is further metabolised to noroxymorphone via CYP2D6 enzymes. The minor metabolic pathways are formation of oxymorphone via CYP2D6 enzymes, and 6-keto reduction to α -/ β -oxycodol. Oxymorphone is further metabolised to noroxymorphone via CYP3A4 enzymes. Adapted from Andreassen *et al.*, 2010

I.6.3 Effects of oxycodone

Oxycodone imparts its analgesic effect by binding to specific CNS OR in the brain and spinal cord. It is a full opioid agonist that binds to KOR, MOR and DOR (Ordonez Gallego *et al.*, 2007). It has more affinity to the KOR than MOR or DOR in this order respectively. Studies suggest that its analgesic action is due to the activation of KOR in contrast to morphine which interacts primarily to MOR (Ross & Smith, 1997). Oxycodone produces analgesia without loss of consciousness. It induces cough suppression by direct action on the cough reflex center in the medulla. Cough suppression occurs at lower doses than generally required for anesthesia.

Oxycodone acts directly on the brain stem respiratory center to produce respiratory depression. Both a reduction in the responsiveness of the brain stem respiratory centers to increase carbon dioxide tension and electrical stimulation are involved in the respiratory depression. Oxycodone causes peripheral vasodilatation, inhibits baroreceptor reflexes and decreases peripheral resistances. Oxycodone may also lead to histamine release and hence it may cause pruritis, flushing, sweating, red eyes and orthostatic hypotension. Like other opioid analgesic, nausea and vomiting is caused by direct stimulation of chemoreceptor trigger zone located in the medulla. It may also cause a decrease in hydrochloric acid secretion in the stomach, resulting in decreased motility.

Oxycodone

Site	Effects
Central nervous system	Analgesia, antitussive, miosis of pupils, respiratory depression.
Cardiovascular system	Orthostatic hypotension
Gastrointestinal tract	Decrease gastric, biliary and pancreatic secretions; decrease gastrointestinal tract motility, constipation, elevation in serum amylase.

Table 7: Oxycodone and some of its effects on important organs in the body.

When oxycodone is used for the relief of moderate to severe pain, it is available in immediate and controlled release dosage forms. Controlled release oxycodone is subject to abuse by drug

addicts who grind the tablets and use it for an immediate high dose effect, causing a sense of euphoria and increasing the possible risk of toxicity. The efficacy of oxycodone may be modified by CYP2D6 polymorphism due to a decrease in the clearance of the drug. However, since its therapeutic effect depends on the parent drug, CYP2D6 polymorphism may not affect the analgesic effect of oxycodone, but it might affect the toxicity profile.

I.6.4 Oxycodone excretion

The kidney is the primary excretion site for oxycodone and its metabolites. The total plasma clearance is approximately 1.4 L/min in adults. Most of oxycodone and noroxycodone is excreted in urine as the free unconjugated form whereas oxymorphone is mainly excreted in the conjugated form (Kalso, 2005).

I.6.5 Oxycodone pharmacokinetics

The pharmacokinetic parameters of oxycodone are shown in Table 7. The volume of distribution of oxycodone is 2-3 L/kg. The $t_{1/2}$ is about 2-3 hours after i.v. administration and about 3 hours after immediate-release solution. The maximum plasma concentration of oxycodone are reached within 25 min after i.v administration and after 1.3 h after immediate-release (Kalso, 2005).

A study reported that clearance of intravenous oxycodone was reasonably high (approximately 0.8 L/min), which was predicted as a medium hepatic extraction and a moderate first-pass effect of oxycodone (Lalovic *et al.*, 2006). The urinary metabolites derived from CYP3A4-mediated N-demethylation of oxycodone (noroxycodone, noroxymorphone, and α - and β -noroxycodol) accounted for $45\% \pm 21\%$ of the dose, whereas CYP2D6-mediated O-demethylation (oxymorphone and α - and β -oxymorphol) and 6-keto-reduction (α - and β -oxycodol) accounted for $11\% \pm 6\%$ and $8\% \pm 6\%$ of the dose, respectively (Lalovic *et al.*, 2006). In infants and neonates a large variability in clearance and $t_{1/2}$ has been reported and hence routine dosing in this population might be dangerous (Pokela *et al.*, 2005). The pharmacokinetics of intravenous oxycodone in children aged 6-93 months are fairly similar to those reported in adults (Kokki *et al.*, 2004). A marked interindividual variation in the pharmacodynamics and pharmacokinetics of oxycodone was observed supporting the need for individualized dosing regimen (Lugo & Kern, 2004)

Species	Drug and dose	AUC (ng/ml/h)	C _{max} (ng/mL)	T _{max} (h)	t _{1/2} (h)	V _{ss} (L/kg)	CL (L/min)	Bioavailability	Reference
Human	IRS oxycodone 20 mg	194 ± 23 (0, 36 h)	41.6 ± 6.1	1.3 ± 0.6	3.2 ± 0.9	-	-	-	(Mandema, <i>et al.</i> , 1996)
Human	IRS oxycodone 0.28 mg/kg	245 ± 84	38 ± 14	-	5.1 ± 1.7	-	-	0.6 ± 0.2	(Poyhia, <i>et al.</i> , 1992)
Human	I.V. oxycodone 0.07 mg/kg	-	-	-	2.3 (2-5.5)	2.6 (2.2-3.0)	0.78 (0.5-1.0)	-	(Pöyhiä, <i>et al.</i> , 1991)
Human	I.V. oxycodone 0.05 mg/kg	70 (52-88)	13 (9-17)	0.42 (0.33-4)	2.6 (2-3.1)	2.0 (1.1 - 2.9)	0.83 (0.6-1.0)	-	(Takala <i>et al.</i> , 1997)
Sprague Dawley rats	I.V. 5 mg/kg	2957 ± 204	-	-	0.7 ± 0.1	2.8 ± 0.2	4.9 ± 0.3	-	(Chan <i>et al.</i> , 2007)
Sprague Dawley rats	I.V. 5 mg/kg	4671 ± 377	-	-	1.1 ± 0.2	2.2 ± 0.2	3.1 ± 0.3	-	(Chan <i>et al.</i> , 2007)
Sprague Dawley rats	Oral 10 mg/kg	69 ± 9	43 ± 8	0.26 ± 0.12	3.0 ± 0.5	-	-	1.2	(Chan <i>et al.</i> , 2007)
Sprague Dawley rats	Oral 10 mg/kg	470 ± 47	215 ± 39	0.11 ± 0.03	4.0 ± 0.5	-	-	5.0	(Chan <i>et al.</i> , 2007)

Table 8: Pharmacokinetics of oxycodone in humans and rats. Adapted from Chan *et al.* 2007 and Kalso, 2005.

I.6.6 Effect of age

Pharmacokinetic of intravenous oxycodone were reported to be dependent on the age of patients. Patients aged 60-70, 70-8- or 80-90 years had 28-34% lower clearance of oxycodone than patients aged 20-40 years. The change in clearance of oxycodone lead to up to 80% higher exposure to oxycodone in the oldest group than in the youngest group. Patients aged above 70 years are expected to have a 40-80% exposure to oxycodone than young adult patients (Liukas *et al.*, 2011).

A decrease in the elimination clearance of oxycodone with advancing age in reflected in the elimination half-life, which increased with increasing age. A significant increase in the time needed for 50% decrease in concentration after continuous infusion of variable length was seen in the elderly. The predicted time course after repeated bolus dosing of oxycodone showed that oxycodone may accumulate in the elderly compared to young adults. The predicted peak concentrations of oxycodone were also much higher in the elderly (Saari *et al.*, 2012).

In a comparative study between patients aged 70-90 years and 20-40 years, the former group had a 50-80% higher mean exposure to oxycodone and a twofold higher plasma concentration than the young adults after 12h ingestion of oral oxycodone. The apparent clearances were lower in the elderly patients than in the 20-40 group. The mean elimination half-life of oxycodone in the 70-90 group was 1.6 h longer than in the youngest group. Age was found to be a significant covariate for oxycodone pharmacokinetics. In elderly patients, dosing should therefore be reduced and carefully titrated to avoid considerable accumulation of oxycodone and potentially hazardous side effects (Saari *et al.*, 2012). These also data demonstrate that the elimination of oxycodone is reduced with advancing age

In healthy volunteers with specific CYP2D6 genotyped and phenotyped, oxycodone analgesic effects were assessed in experimental pain tests. Differences depending on CYP2D6 activity was demonstrated in the analgesic response, UM experienced a 1.5- to 6-fold increase of the analgesic effects as compared with EM. PM had a 2–20-fold reduction of the effects as compared with EM. This is in agreement with the few case reports describing reduced analgesic effect of oxycodone in PM.

Differences according to CYP2D6 phenotype were similarly demonstrated in other assessed pharmacodynamic parameters (pupil size, saturation, sedation and psychomotor effects). Oxycodone had no significant effect on the pupil size of the genotypic PM whereas it induced a pupil constriction in all other volunteers. It had no effect on the sedation and saturation assessment in PM, whereas they were higher in UM than EM (Samer *et al.*, 2010). Oxycodone treatment *pro re nata* was intolerated by an elderly patient who was previously noted with a long-standing intolerance to codeine, patient showed significant side effects with the drug (Susce *et al.*, 2006). The pharmacokinetics of oral oxycodone were seen to depend upon the age of the patients and their CYP2D polymorphism.

Likely phenotype	Genotypes	Recommendations for oxycodone therapy
Ultrarapid metabolizer	An individual carrying more than two copies of alleles.	Use alternative drug to oxycodone, not codeine or tramadol or be alert to adverse drug events.
Extensive metabolizer	An individual carrying two alleles encoding full function allele together with reduced-function allele.	Use label recommended age or weight-specific dosage.
Intermediate metabolizer	An individual carrying one reduced and one non-functional allele.	Use alternative drug to oxycodone not codeine or tramadol or be alert to insufficient pain relief.
Poor metabolizer	An individual carrying no functional alleles.	Use alternative drug to oxycodone not codeine or tramadol or be alert to insufficient pain relief.

Table 9: Recommended dosage of oxycodone by CYP2D6 phenotypes. Adapted from Swen *et al.*, 2011.

I.7 Physiological and pharmacokinetic effects of aging

Aging is a normal physiological phenomenon characterized by the progressive degeneration of organ systems and tissues. It has been defined as a complex multifactorial process and it is associated with numerous declines in physiological and cognitive functions that lead inevitably to death. Environmental factors such as diet, exercise, pollutants, exposure to radiation and pathogenic microorganisms influence the process of aging. Gender also plays a role in aging; in most developed countries women tend to outlive men by 7 to 10 years. Another factor that can influence aging is the individual's genetic make up. The process of aging falls into three

categories with advancing age; changes in cellular homeostatic mechanisms, decrease in organ mass and decline and loss of functional reserve of the body's system.

There are various theories related to aging. The first interesting category of theories is that aging is 'programmed', where delineated biological alterations in homeostasis and natural defence will take place over time. The second category is the 'error' theory where it is assumed that the body undergoes progressive damage due to environmental insults. The third "free radical theory" suggests that reactive oxygen species generated during mitochondrial energy production are damaging because of free radicals with evidence of oxidative change to DNA, protein and lipids.

Changes with age occur in every individual but not necessarily at the same rate for each organ and tissue. In the cardiovascular system, age leads to a decrease in elasticity and an increase in stiffness of the arterial system, resulting in an increase in the afterload on the left ventricle, an increase in the systolic blood pressure and left ventricular hypertrophy (Chen *et al.*, 1998; Vaitkevicius *et al.*, 1993). Furthermore the observed drop out in the pacemaker cells leads to a decrease in the intrinsic heart rate. In addition to that a decrease in the responsiveness to adrenergic receptor stimulation, decreases reactivity to baroreceptors, chemoreceptors and an increase in catecholamines which lead to all the diseases related to cardiovascular system (Cheitlin, 2003). These cardiovascular diseases seen in the elderly include, to name a few isolated systolic hypertension, diastolic dysfunction, heart failure, atrioventricular conduction defects and aortic valve calcification. The lungs are also affected with aging which causes the decrease in peak airflow and gas exchange, the decrease in vital capacity, the weakening of respiratory muscles and the decline in the effectiveness of lung defense mechanism.

Aging has always been closely linked with a decrease in the renal elimination of drugs. The changes that take place in the kidney with age include a decrease in renal mass that reflects a reduction in nephrons, a decline in renal plasma flow and glomerular filtration rates and an average of 8mL/min/1.73cm²/decade decline in creatinine clearance after the age of 30 in humans (Dunnill & Halley, 1973; Glasscock & Winearls, 2009; McLachlan, 1978). The decrease in creatinine clearance does not lead to a concomitant increase in plasma creatinine due to the related loss of muscle mass with age (Mangoni & Jackson, 2004).

Hepatic metabolism of drugs by Phase I reactions is likely to be prolonged in the elderly (Klotz, 2009) whereas no significant reduction in Phase II metabolism was observed (Le Couteur *et al.*,

2002). For drugs with decreased metabolism, clearance typically decreases by 30 to 40%. Theoretically, drug dose should be decreased by the same percentage, but the rate of drug metabolism decline, varies between different individuals. . First pass metabolism of drugs, decrease with age, probably, due to the decrease in liver mass and blood flow, hence increasing the bioavailability of drugs (Mangoni & Jackson, 2004), but the bioavailability of prodrugs that require activation by the liver also decreases. A change in the absorption, distribution, metabolism and elimination of drugs is observed with age, with some changes having more profound effects on drugs than others. The metabolism and excretion of many drugs are known to decrease with age leading to prolongation of half life of drugs in the body (Mangoni & Jackson, 2004), hence increasing the systemic exposure of the body to the drug which might lead to toxicity and side effects.

I.7.1 Absorption

In elderly patients, a decrease in gastric emptying, an increase in pH and a decrease in small-bowel surface area tend to be clinically inconsequential for most drugs with the exception of a few drugs where optimum pH is required, for example, for the absorption of calcium-carbonate. Pharmacokinetic studies on absorption in relation to age provide conflicting results. Although absorption does not seem to be affected for most drugs, supplements like vitamin B12, iron and calcium that require active transport across the intestine are reduced (Blechman & Gelb, 1999), whereas absorption of drugs like levodopa is increased perhaps due to a decrease in dopa decarboxylase (Klawans HL, 1990).

I.7.2 Distribution

It is known that with age there is a decrease in lean body mass, an increase in body fat and a decrease in total body water (Shi & Klotz, 2011). This leads to an increase in the volume of distribution of highly lipophilic drugs that may lead to an increase in their elimination half-life and volume of distribution. Polar drugs that are water-soluble tend to have smaller volume of distributions and hence higher serum levels in elderly patients (Cusack *et al.*, 1979; Redolfi *et al.*, 1979; Vestal *et al.*, 1977). The reduction in the volume of distribution of water-soluble drugs in the elderly tends to be balanced out by a reduction in renal clearance with a very low effect on the half life of the drugs (Mangoni & Jackson, 2004).

I.7.3 Metabolism

Clearance of drugs by the liver depends on the capacity of the liver to extract drugs from the blood passing through it and the hepatic blood flow rate. The formula:

$$CL_{\text{liver}} = \frac{Q (C_a - C_v)}{C_a} = QE$$

where E= steady state extraction ratio, Q=liver blood flow (sum of hepatic portal and hepatic arterial blood flow), C_a = concentration of drug in portal vein and hepatic artery, C_v = concentration of drug leaving the liver in the hepatic vein and CL_{liver} = clearance by the liver depicts that clearance depends on the blood flow and the extraction ratio. Extraction ratio is dependent on the metabolizing capacity of the liver; in other words it is dependent on drug metabolism by the liver.

The liver contains a large number of enzymes but the family of enzymes responsible for most of the drug metabolism are the CYP450 (Phase I) out of which the sub families CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 play an important role in drug metabolism (Cupp & Tracy, 1998). The main aim of the body in metabolizing drugs is to convert the drugs to more polar compounds that can be readily excreted from the body. Interestingly when these enzymes metabolize drugs they can produce active or inactive metabolites. If the metabolites formed are inactive the dose of the drug that reaches the systemic circulation is reduced whereas if the metabolites formed are active then depending on the enzyme activity and the amount of active metabolite formed the efficacy of the drug will vary. CYP3A4 is said to metabolize almost 50% of the drugs currently present in the market and another 30% of drugs are metabolized by CYP2D6 (Zuber *et al.*, 2002).

“Reduction in hepatic blood flow and in hepatic sinusoidal endothelium with effects on drug transfer and oxygen delivery, reduce hepatic drug clearance” (McLean & Le Couteur, 2004). Reduced liver mass in aged patients may be responsible for the reduced clearance of many agents. There is a significant reduction in the clearance of many drugs metabolized by Phase I pathways in the liver. It remains unclear whether enzyme responsiveness changes with aging in humans. No major effects of aging in the pathways of conjugation were reported.

Differences in terms of pharmacodynamics and therapeutic drug response due to the influence of age and gender on the activity of hepatic and gastro intestinal CYP3A4 isoforms have yielded inconsistent results (Cotreau *et al.*, 2005). Studies on the effect of age on triazolam, an exclusive CYP3A4 substrate, demonstrated significant decrease in weight-normalized clearance among healthy elderly subjects compared to young control subjects (Greenblatt *et al.*, 1983; Greenblatt *et al.*, 1991). Other reports state that the effect of age is not significant (Robin *et al.*, 1996; Smith *et al.*, 1983). A study that involved S9 fractions from Sprague Dawley male rats of age groups 3-, 6-, 12- and 18-month old showed that the pharmacokinetic parameters ($t_{1/2}$ and AUC) increased and drug clearance of ketamine-xylazine decreased with aging. The findings for liver S9 fractions of 18-month-old rats compared with the other age groups suggested that following a normal ketamine anesthetic dose, drug metabolism was impaired, leading to significant increase of drug exposure (Giroux *et al.*, 2013).

I.7.4 Elimination

Reduction in renal function in elderly patients affects many drugs such as water-soluble antibiotics (Lumholtz *et al.*, 1974; Triggs *et al.*, 1980), diuretics (Somogyi *et al.*, 1990), digoxin (Portnoi, 1979), β -adrenoreceptor blockers (Rigby *et al.*, 1985), lithium (Hewick *et al.*, 1977) and NSAIDs (Oberbauer, 1993; Ritch, 1982). Reduction of renal elimination is clinically important because it can lead to toxicity of drugs due to their accumulation in the body.

I.8 Age-related changes on pharmacodynamics of drugs

Biological aging processes are linked mechanistically to altered drug handling, altered physiological reserve and pharmacodynamics responses. Oral loading doses may require a reduction in account for age-related increase in bioavailability. Altered pharmacodynamics are well documented in the cardiovascular system, with changes in the autonomic system, autacid receptors, drug receptors, and endothelial function to modify baseline cardiovascular tone and responses to stimuli such as postural change and feeding (McLean & Le Couteur, 2004).

Older adults show an exaggerated response to CNS active drugs. It is in part due to an underlying decline in CNS function and increased pharmacodynamics sensitivity for some

benzodiazepine, anesthetic and opioid drugs (Bowie & Slattum, 2007). Shorter duration and a decreased depth of anesthesia with aging was reported in a study involving 3-, 6-, 12- and 18-month old Sprague Dawley rats. Significant cardiac and respiratory depression, as well as decreased blood oxygen saturation, occurred in all age groups however, cardiac frequency was the most affected parameter with aging (Giroux *et al.*, 2015). Data regarding the effect of age on opioid pharmacokinetics and pharmacodynamics are scarce and in some cases the results have shown conflicting conclusions.

A decrease in renal weight and number of glomeruli in subjects older than 50 years due to aging process is observed. The basement membrane of the kidneys thickens and the number of sclerotic glomeruli increase. These changes occur due to normal aging but can be accelerated by onset of diseases (Muhlberg & Platt, 1999). Renal aging is evident through reduction in renal clearance in the elderly that is primarily disease-related. Pathophysiology plays an increasingly important role with age and thus may also affect the pharmacodynamics of drugs. Polypharmacy, the administration of various drugs to a single patient is common in the elderly and it increases the risk of drug interactions and side effects. Polypharmacy and adverse drug reactions represent major links to avoidable morbidity and mortality.

A general trend of greater pharmacodynamics sensitivity is observed in the elderly however it is not universal and the age-related changes must be investigated agent by agent until further research yields greater understanding of the molecular mechanisms underlying the aging process.

I.9 Effects on polymorphism and aging

Pharmacokinetics and pharmacodynamics changes occur with increasing age. Several studies have shown a decrease in the metabolism of drugs by CYP450 with age. The clearance of theophylline by CYP1A2 was decreased with aging in humans (Jusko *et al.*, 1979; Ohnishi *et al.*, 2003; Otero *et al.*, 1996; Vestal *et al.*, 1993). This has been interpreted as representing a decrease in CYP1A activity with aging (Schwartz, 2007).

Limited data is available on age related changes on metabolism of CYP2C9 and CYP2C19 substrates in humans, but the studies that have been performed shows age related decrease in clearance in the order of 25% and show slower clearance in women compared to men

(Bachmann & Belloto, 1999). After a single intravenous dose, the effects of genotype and age were analyzed for omeprazole. CYP2C19 pharmacogenetic variants were significant covariates, for systemic clearance, the elderly extensive metabolizers showed wide variation in clearance and were phenotypically closer to the elderly poor metabolizers, than the young extensive metabolizers to the young poor metabolizers. The conclusion was CYP2C19 genotyping might not be as useful as phenotyping in the elderly (Ishizawa *et al.*, 2005).

Genetic polymorphism of CYP2D6 results in varying clearance ratios for its substrates in humans. Dextromethorphan metabolic ratios have been reported as slightly higher in older women than in middle aged women suggesting slower metabolism in elderly (O'Connell *et al.*, 2006). In healthy subjects with either extensive metabolizer phenotype or genotype, men have been reported to have faster clearance of dextromethorphan than women (Labbe *et al.*, 2000; Tamminga *et al.*, 1999). In Japanese men with extensive or intermediate metabolizer genotype, older age was also associated with slower haloperidol clearance (Ohara *et al.*, 2003). Literature review on substrate clearance by CYP2D6 in humans suggests that older age is associated with slower clearance on the magnitude of 20% (Dorne *et al.*, 2002), suggesting that doses of CYP2D6 substrates should be reduced by approximately 20% in the elderly (Schwartz, 2007).

Rodent models have shown a consistent decrease in clearance of CYP3A substrate with aging (Santamaria R. *et al.* 2015, Cotreau *et al.*, 2005; Patki *et al.*, 2004; Schmucker, 2001) but this has not been observed in humans (Cotreau *et al.*, 2005; Patki *et al.*, 2004; Schmucker, 2001). *In vitro*, using human liver microsomes, earlier studies have shown no decrease in CYP3A activity or content with age (Parkinson *et al.*, 2004; Schmucker *et al.*, 1990). CYP3A activity and expression in non-alcoholic fatty liver disease were recently evaluated and it was observed that *in vivo* CYP3A activity was reduced (Woolsey *et al.*, 2015). Liver fibrosis was associated with lower CYP3A enzyme function. CYP3A mRNA expression *in vivo* was found to be reduced in non alcoholic fatty liver disease than in the normal healthy control subjects leading to a decrease in CYP3A activity (Woolsey *et al.*, 2015).

I.10 *In vitro* metabolism models

The most effective approach to determine *in vivo* metabolism in humans is by *in vitro* screening using liver-derived experimental systems. Over the decades many *in vitro* liver models have

been developed and they include tests that use: primary hepatocytes, liver slices, perfused liver, cytosol, liver cell lines, transgenic liver cell lines, microsomes, liver S9 fractions, supersomes.

With the exception of perfused liver, liver slices, and primary hepatocytes, all the in-vitro models require exogenous cofactors for their activities. The two exogenous factors used are NADPH (Nicotinamide adenine dinucleotide phosphate) system for Phase I oxidation, essential for measurement of oxidase activity catalyzed by CYP450 or uridine-5'-diphospho-alpha-D-glucuronic acid (UDPGA) responsible for Phase II glucuronidation.

I.10.1 Hepatocytes

Hepatocytes are used to study both Phase I oxidation process and Phase II glucuronidation process in the liver (Li, 2005). They have an excellent *in vivo-in vitro* correlation. Their major disadvantage is that they lack non-hepatocyte cells and hence there is no cofactor supply. Furthermore there can be individual variations, which can be reduced by using hepatocyte pools from multiple donors (Brandon *et al.*, 2003). Primary hepatocytes are used for metabolic stability studies, for identification of metabolites and their profiling, for drug-drug interactions and hepatotoxicity studies.

I.10.2 Liver slices

Liver slices are not available commercially (Brandon *et al.*, 2003). They are normally used to study induction of CYP isoforms. Nutrient media cannot easily penetrate into the liver slices and hence CYPs have a very short viability of 5 hours, which is a disadvantage.

I.10.3 Perfused liver

Perfused liver can only come from animals and not from humans. The method is labour intensive, has poor reproducibility and its viability lasts only 3 hours (Brandon *et al.*, 2003). It is only used when bile secretions are necessary for metabolic studies or when validation of another *in vitro* model is required.

I.10.4 Cytosol

Cytosol is the aqueous part of the cytoplasm consisting of water, organic solvents and ions. Cytosol contains Phase II enzymes like glutathione S-transferase, N-acetyl transferase, and sulfotransferase. It is commercially available. The advantage of cytosol is that soluble Phase II enzyme metabolism can be investigated but enzymes like UGT (Uridine 5'-diphosphoglucuronyltransferase) which are normally present on the endoplasmic reticulum (ER) cannot be investigated.

I.10.5 Cell lines

Cell lines are used only in enzyme induced states or in the study of cytotoxicity of drugs. In cell lines there is an incomplete expression of metabolic enzymes. Cell lines that are commercially available include Hep G2 from hepatocellular carcinoma, BC2 from hepatoma etc. Since cell lines have low expression or absence of Phase I and Phase II enzymes, they are not used for studies of CYPs metabolism (Asha & Vidyavathi, 2010).

I.10.6 Transgenic cell lines

Cell lines are transfected at high efficiency using centrifugation of lysozyme-treated bacteria bearing the desired vector with the parent cells in the presence of polyethylene glycol protoplast fusion (Brandon *et al.*, 2003). Transgenic cell lines are very expensive and are generally used to form metabolites for structure elucidation. They have high expressions of CYPs and UGT and they can be used to study single enzyme reactions. They do not provide complete information of *in vivo* metabolism due to the presence of some isozymes.

I.10.7 Microsomes

These are prepared by homogenization of the liver followed by centrifugation of the subcellular fraction from ER of hepatic cells (Pelkonen *et al.*, 1974). Microsomes are excellent for the study of Phase I reactions. NADPH system is added to provide energy as a cofactor for the CYPs, otherwise UDPGA can be added for glucuronidation reactions. Microsomes are rich in CYPs,

carboxylesterase, flavin monooxygenase and a few other enzymes. The main advantages of microsomes include low cost, easy storage, possibility of gender study and possibility of studying inter individual variations. The disadvantages of microsomes include high concentration of CYPs and hence it does not resemble *in vivo* metabolism where the concentration of enzymes is lower, Phase II enzymes are absent and so are the cytosol enzymes, incubation of microsomes with different solvents, pH values and different chemicals can affect the metabolism and formed metabolites. There are inter-individual variations in the activity of human liver microsomes, but this problem can be overcome by the application of pooled microsomes (Araya & Wikvall, 1999).

I.10.8 Liver S9 fractions

S9 fractions have both microsomal and cytosolic fractions of the liver and hence they are able to give complete profile of the metabolism that can occur *in vivo* in intact liver cells. NADPH system has to be added to supply CYPs with energy required by them to metabolize xenobiotics. Phase I and Phase II enzymes are all present and hence all metabolites formed by these enzymes can be studied. S9 fractions have lower expression of enzymes compared to cytosol and microsomes, hence some metabolites might go undetected. For the catalytic activity of phase II enzymes, addition of exogenous cofactors is necessary: UDPGA and alamethicin for UGT; acetyl CoA, DTT, and acetyl CoA regenerating system for N-acetyl transferase; adenosine-3-phosphate-5-phosphosulphate for sulfotransferase; and glutathione for glutathione-S-transferase (Brandon *et al.*, 2003). A more complete representation of metabolic profile in the liver is offered by S9 in comparison to cytosol and microsomes. Metabolites formed by phase I reaction followed by Phase II reaction might not be formed in cytosol and microsomes but can be detected with S9 fractions.

I.10.9 Supersomes

Microsomes, which consist of vesicles of the hepatocyte endoplasmic reticulum of human CYP- or UGT-transfected insect cells are referred to as baculosomes or supersomes (Chen *et al.*, 1997). Supersomes have specifically expressed human CYPs and UGTs. It allows the investigation of the contribution of a single metabolic enzyme to the biotransformation pathway of the compound under investigation. All common human CYPs, coexpressed with NADPH-cytochrome P450

reductase and UGT are available in supersomes. The use of a regenerating NADPH-system is required to supply energy for UGT, thus uridine diphosphate glucuronic acid has to be added as cofactor. The advantages of supersomes include their use to study isozyme-specific drug transformation, drug to drug interactions, and the influence of polymorphism on the drug biotransformation pattern. The disadvantages in UGT supersomes are the UGT active site is shielded behind a hydrophobic barrier, which can be overcome by using a pore-forming agent (Fisher *et al.*, 2001).

I.11 Substrate depletion model

The prediction of *in vivo* drug clearance from *in vitro* drug metabolite-kinetic data is well established in the rat. The metabolic rate-substrate concentration relationship can commonly be described by the classic hyperbola consistent with Michaelis-Menten model. A wide variety of drugs and other foreign compounds are metabolized by CYPs. The kinetic properties of these enzymes are often described satisfactorily by the classical Michaelis-Menten model:

$$V = \frac{V_{max} \times S}{K_m + S} \quad (1)$$

V = velocity of the metabolic reaction, S = substrate concentration, K_m = Michaelis Menten constant; when the rate of metabolism is 50% of V_{max} .

Michaelis-Menten model can be used for scaling *in vitro* kinetic data to predict the *in vivo* clearance of drugs (Houston, 1994). Therapeutic drug concentrations rarely approach the K_m ; the first order limit of equation (1) is applicable to describe the rate of metabolism *in vivo*. At very low substrate concentration, when [S] is much less than K_m , equation (1) is formed, the rate is directly proportional to the substrate concentration.

$$\frac{V}{S} = \frac{V_{max}}{K_m + S} \approx \frac{V_{max}}{K_m} \quad (1)$$

The determination of *in vitro* intrinsic clearance (CL_{int}) for drug candidates in the early drug discovery stage is a common practice in the pharmaceutical industry (Houston, 1994; Lave *et al.*,

1997; Obach *et al.*, 1997). The CL_{int} values of drug candidates can help to confirm whether metabolism is the main clearance pathway when it is compared with the total body clearance *in vivo*. The *in vitro* CL_{int} may be derived from enzyme kinetic data such as V_{max}/K_m (Lin *et al.*, 1996; Tan and Pang, 2001; Griffin and Houston, 2004) or from the *in vitro* $t_{1/2}$ values where sub K_m substrate concentrations are used (Lave *et al.*, 1997; Obach 1999; Lau *et al.*, 2002; Jones and Houston, 2004). The ratio V_{max}/K_m , provides the parameter intrinsic clearance (CL_{int}) which defines the rate of metabolism for a given drug concentration.

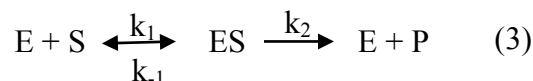
$$CL_{int} = \frac{V_{max}}{K_m} \quad (2)$$

An important parameter for CYP450-mediated drug biotransformation reaction is the Michaelis constant (K_m). Enzyme kinetic parameters; K_m , V_{max} and CL_{int} for CYP450 catalyzed reactions is an important aspect in drug discovery and development (Obach, 2001). These *in vitro* data can be used to optimize the human pharmacokinetic behavior of new chemical entities prior to human administration (Obach & Reed-Hagen, 2002).

K_m is an indicator of the affinity that an enzyme has for a particular substrate, hence the thermodynamic stability of the enzyme- substrate complex. The stability of the enzyme-substrate complex is closely related to the enzyme structure. It plays a central role in defining energetically favored binding cluster of the substrate in the active enzyme site (Lavoie *et al.*, 2013). K_m value is a primary descriptor of enzyme kinetic behavior of biotransformation reaction. It represents the substrate concentration that will yield a reaction velocity that is half of the theoretical maximum velocity that would occur at an infinite substrate concentration (V_{max}). In other words, the K_m value tells us the potential of a drug to saturate or partially saturate an enzyme involved in its metabolic clearance. Lower K_m values show a greater possibility that the drug concentration attained *in vivo* will saturate the metabolic clearance pathway.

The rate of metabolite formation at several substrate concentrations can be assessed for the conventional determination of K_m values. Substrate loss versus time using the *in vitro* $t_{1/2}$ approach gives overall intrinsic clearance estimates. It involves the measurement of substrate consumption at a single low concentration ($<K_m$). This simple design yields an estimate of intrinsic clearance.

K_m provides a measure of the substrate concentration required for significant catalysis to occur. Experimental evidence shows that K_m for many enzymes provides an approximation of substrate concentration in vivo. K_m is also related to the rate constant of the individual steps in the catalytic scheme:



$$K_m \text{ is defined as } \frac{k_{-1} + k_2}{k_1} \quad (4)$$

If $k_{-1} \gg k_2$ the ES complex dissociates to E and S much more rapidly than the product is formed

$$K_m = \frac{k_{-1}}{k_1} \quad (5)$$

The dissociation complex is given by:

$$K_{ES} = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} \quad (6)$$

Therefore K_m is equal to the dissociation constant of the ES complex if $k_2 \ll k_{-1}$. In such cases, K_m is a measure of the ES complex; a high K_m indicates weak binding; a low K_m indicates strong binding. V_{max} indicates the turnover number of an enzyme, which is the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate. It is equal to the kinetic constant k_2 , also known as k_{cat} . A decreased V_{max} value suggests a rapid saturation of enzyme active sites that can be due to the formation of a more stable enzyme-substrate complex (ES), leading to a decrease of k_{cat} and V_{max} due to an increase in ΔG^\ddagger .

$$k_{cat} = \frac{V_{max}}{[ET]} \quad (7)$$

I.12 Animal Cytochrome P450

The use of animal models for preclinical drug development for the prediction of metabolic behaviour in humans requires a connection between the different isoforms of CYPs present in humans to animals. Pharmacokinetic data can be extrapolated from animals to humans

reasonably well applying appropriate pharmacokinetic principles. There is a relatively small difference in the primary amino acid sequences of CYPs across species since they possess a highly conserved region of amino acid residues. However, even small changes in the amino acid sequences can give rise to profound differences in substrate specificity and catalytic activity (Martignoni *et al.*, 2006). Table shows the different CYP isoforms present in animals and humans.

CYP2D6. In human CYP2D accounts for only 4% of the total CYPs present in the liver (Madani *et al.*, 1999; Zuber *et al.*, 2002) and only one isoform CYP2D6 is expressed whereas in rats six isoforms have been identified (Nelson *et al.*, 1996). The rat and human CYP2D isoforms have a high sequence identity (>70%) (Venhorst *et al.*, 2003). Among the six isoforms CYP2D1 is the rat orthologue of human CYP2D6 (Martignoni *et al.*, 2006). Other studies state that rat CYP2D2 is functionally conserved with human CYP2D in endogenous morphine formation (Grobe *et al.*, 2012). Like humans, polymorphism in rats has also been documented to provide models for PM and EM in humans (Al-Dabbagh *et al.*, 1981).

CYP3A. The content of CYP3A in the human liver accounts for only 30% of total CYPs present. Four isoforms of CYP3A are expressed in humans: CYP3A4, A5, A7 and A8. Of these CYP3A4 and CYP3A5 are the most abundant. In rats six isoforms have been reported that include CYP3A1, A2, A9, A18, A23 and A62. CYP3A2 in rats exhibits a 73% homology of the amino acid sequence and some substrate preference as well as functional analogies to human CYP3A4 (Martignoni *et al.*, 2006).

Family	Subfamily	Human	Mouse	Rat	Dog	Monkey
CYP1	A	1A1, 1A2	1A1, 1A2	1A1, 1A2	1A1, 1A2	1A1, 1A2
	B	1B1	1B1	1B1	1B1	1B1
CYP2	A	2A6, 2A7, 2A13	2A4, 2A5, 2A12, 2A22	2A1, 2A2, 2A3	2A13, 2A25	2A23, 2A2A
	B	2B6, 2B7	2B9, 2B10 2C29, 2C37,	2B1, 2B2, 2B3	2B11	2B17
	C	2C8, 2C9, 2C18, 2C19	2C38, 2C39, 2C40, 2C44, 2C50, 2C54, 2C55	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23	2C21, 2C41	2C20, 2C43
	D	2D6 , 2D7, 2D8	2D9, 2D10, 2D11, 2D12, 2D13, 2D22, 2D26, 2D34, 2D40	2D1 , 2D2 , 2D3, 2D4, 2D5, 2D18	2D15	2D17, 2D19, 2D29, 2D30
	E	2 E1	2 E1	2 E1	2 E1	2 E1
CYP3	A	3A4 , 3A5, 3A7, 3A43	3A11, 3A13, 3A16, 3A25, 3A41, 3A44	3A1/A23 , 3A2, 3A9, 3A18, 3A62	3A12, 3A26	3A8

Table 10: Major drug metabolizing CYP family of enzymes in humans, rat, mouse, dog and monkey. The isoforms in red represent the rat analogs to the human isoforms. Adapted from Martignoni *et al.*, 2006.

I.13 Common probe substrates

The study of the metabolism of CYP450 enzymes by CYP probe substrates is well established in literature. Specific substrates for each subfamily of the CYP450 are well documented. The assessment of metabolic activity of various CYP enzymes is mostly assessed using selective substrates of distinct CYP enzymes that is a drug which is ideally metabolized by single CYP enzymes (Jurica *et al.* 2012). Pharmacokinetic of ideal marker should be determined by metabolism and not by intensity of liver perfusion, protein binding or elimination of unchanged drug.

CYP2D6. Probe substrates for CYP2D6 are listed in Table 8. Among them dextromethorphan and debrisoquine are the most preferred for *in vitro* preclinical studies. The use of tramadol as a probe substrate is limited because *in vitro* studies show that the metabolite O-demethyltramadol is also mediated by CYP2B6 at a high extent.

CYP3A4. Common probes for CYP3A4 are listed in Table 8. None of the suggested probes for CYP3A4 is metabolized uniquely by this enzyme and the active site is thought to be large and able to bind multiple substrates simultaneously, it is recommended to use at least two structurally unrelated probe substrates for precise enzyme activity evaluation (Jurica et al. 2012).

Enzyme	Probe	Substrate	Enzyme	Probe	Substrate
CYP1A2	Caffeine	N-demethylation	CYP2C19	Chloroguanine	Conversion to cycloguanine
	Phenacetin	O-deethylation		Omeprazole	5-hydroxylation
	Theophylline	N-demethylation		S-mephenytoin	4'-hydroxylation
CYP2C9	Diclofenac	4'-hydroxylation	CYP3A4	Alfentanil	Demethylation
	Losartan	Oxidation		Alprazolam	4-hydroxylation
	Phenytoin	4'-hydroxylation		Codeine	O-demethylation
	S-flurbiprofen	4'-hydroxylation		Cortisol	6- β hydroxylation
	S-warfarin	7-hydroxylation		Dapsone	N-hydroxylation
	Tolbutamide	Methylhydroxylation		Dextromethorphan	N-demethylation
CYP2D6	Codeine	O-demethylation		Erythromycin	N-demethylation
	Debrisoquine	4-hydroxylation		Lidocaine	N-deethylation
	Dextromethorphan	O-demethylation		Midazolam	1-hydroxylation
	Metoprolol	α -hydroxylation		Nifedipine	Oxidation
	Sparteine	Dehydrogenation		Quinidine	3-hydroxylation/ N-oxidation
	Tramadol	O-demethylation	Testosterone	6- β hydroxylation	

Table 11: Probes for CYP450 metabolic activity assessment. Adapted from Jurica J and Sulcova A, 2012.

II Hypothesis and objectives

CYP2D and CYP3A enzymes are involved in the metabolism of a large number of pharmaceutical drugs used in human and veterinary medicine. Nevertheless the effect of age on the metabolic function of these enzymes has not been extensively investigated. A large variation in the pharmacokinetic and pharmacodynamic response to codeine and oxycodone made us hypothesize that a decrease in the metabolic function of these enzymes, causing impaired metabolism of opioids leads to a decrease in the rate of formation of active metabolites and the accumulation of parent drug in the body.

The objectives of this study were

- To study the effect of age on CYP2D metabolic function. For this liver S9 fractions from 3, 6, 12 and 18 month-old rats were used to perform *in vitro* metabolism of codeine and oxycodone.
- To develop an HPLC-MS/MS method for the detection of codeine, oxycodone and their metabolites. An isotopic dilution method was used to quantify the rate of formation of metabolites by CYP2D and CYP3A at different age groups.
- To perform metabolic stability studies of codeine and oxycodone. Michaelis-Menten equation was used to fit the data obtained and evaluate the impairment of CYP function with age using pharmacokinetic parameters.

III Article

Biomedical Chromatography, Accepted

Manuscript online: 7th July 2016

Doi: 10.1002/bmc.3786

***In Vitro* Metabolism of Specific CYP2D and CYP3A Opioid Substrates using Rat Liver S9 Fractions and Mass Spectrometry Reveal a Severe Metabolic Impairment with Increasing Age**

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Keywords: Opioids, Drug Metabolism, Cytochrome P450, Aging, Mass spectrometry

Abstract

Codeine and oxycodone are opioids used to alleviate pain. The outcome of the treatment is ultimately related to their metabolism by Cytochromes P450 (CYPs). Depending on the drugs used, alterations in the metabolism of drugs by CYPs can lead to severe consequences including alterations in their efficacy, safety and toxicity. The objectives of this study were to develop a novel HPLC-MS/MS method capable of quantifying codeine and oxycodone along with specific metabolites using an isotopic dilution strategy and study the rate of formation of morphine (CYP2D), norcodeine (CYP3A), oxymorphone (CYP2D) and noroxycodone (CYP3A). The chromatographic separation was achieved using a Biobasic C18 100 x 1 mm column combined with an isocratic mobile phase composed of methanol and 10 mM ammonium acetate (40:60) at a flow rate of 75 μ L/min. The mass spectrometer was operating in scan mode MS/MS and the analytical range was set at 10–10 000 nM. The precision (%RSD) and accuracy (%RE) observed were 4.4–11.5 and -9.1–6.1% respectively. Liver S9 fractions from 3-, 6-, 12-, and 18-month-old male Sprague Dawley rats were prepared and Michaelis Menten parameters were determined. The derived maximum enzyme velocity (V_{max}) suggested a rapid saturation of the CYP2D and CYP3A active sites in the liver S9 fractions of 18 month-old rats. Moreover, metabolic stability of codeine and oxycodone in rat liver S9 fractions were significantly greater for the 18-month-old rats. This study suggests that there is an impairment of CYP2D and CYP3A metabolism in aging rats.

Introduction

In recent years, the improvements in medicine, and changes in life style have increased life expectancy, and consequently there is a significant increase in geriatric patients needing medical and pharmacological treatments [Bunker., 2001]. A large proportion of marketed drugs have not been thoroughly tested in geriatric or pediatric patients and this has led to numerous medical complications [Richardson *et al.*, 2014; Crensil *et al.*, 2014; Hines, 2013]. Older patients have higher risks of suffering from adverse drug effects which may be associated with a decreased drug clearance (CL) caused by physiologic changes associated with aging [Meziere *et al.*, 2013; Woodhouse *et al.* 1988]. Additionally, other studies show a significant increase in terminal half-life ($T_{1/2}$) of many drugs related to aging [Iirola *et al.*, 2012; Shi and Klotz, 2011] leading to important differences in pharmacokinetic profiles [Veilleux-Lemieux *et al.*, 2013, Giroux *et al.*, 2016]. Alterations in pharmacokinetic and drug metabolism during aging have severe consequences particularly in pain management [Meziere *et al.*, 2013]. The management of chronic pain in elderly patients is certainly not trivial and the efficacy of analgesics are inconsistent, resulting in a diminished quality of life for many patients.

Opioids are important for the clinical management of pain, especially for cancer pain, surgical pain, and they are widely used for the management of chronic pain [Chau *et al.* 2008; Rosenblum *et al.* 2008]. Understanding the metabolism of opioids is therefore important for primary care clinicians. It is well established that the knowledge about opioid metabolism is essential for safety considerations in older and medically complicated patients, who might be taking multiple medications and suffer from inflammation as well as renal, hepatic and immune impairments. Interestingly, a large proportion of opioid drugs are primarily metabolized by CYP2D6 and CYP3A4 with the reported contribution of CYP2B6, CYP2C8, CYP2C9 and CYP2C19 [Smith. 2009]. It is also important to understand that many opioid metabolites contribute significantly to their analgesic effects [Smith. 2009]. Therefore, understanding the fundamental mechanisms

influencing the metabolic fate of opioids is a necessary step for using them safely and efficiently in special population groups (i.e. pediatric and geriatric patients) [Zernikow B et al., 2009; Ginsburg et al., 2009].

Codeine is used for mild to moderate pain treatment whereas oxycodone, which is twice as potent as morphine, is used to treat moderate to severe pain [Curtis GB et al. 1999; Waite I, 2006]. Oxycodone has a lower renal clearance than codeine making it more suitable than morphine for geriatric patients [Ginsburg et al., 2009]. Both codeine and oxycodone exert their effects through μ -opioid receptors located in the central nervous system [Vallejo et al., 2011]. The metabolism of codeine and oxycodone is widely described in the scientific literature and both are substrates of CYP2D6 and CYP3A4 [Lalovic et al. 2004]. Codeine has no significant analgesic effect; it is only through its bioactivation by CYP2D6 leading to morphine that it can impart its analgesic effect [Poulsen L. et al. 1996]. An equivalent mechanism occurs in rats where codeine is converted to morphine by CYP2D1 [Martignoni et al. 2006, Cleary et al., 1994]. Codeine is also a substrate for CYP3A4 forming norcodeine, an inactive metabolite. Lately, oxycodone has widely replaced morphine in the treatment of moderate to severe pain since it has a higher oral bioavailability when compared to morphine [Biancofiore, 2006]. In humans, oxycodone is metabolized by CYP2D6 and CYP3A4 to form oxymorphone and noroxycodone respectively [Löfdal et al. 2013] but in rats it is metabolized by CYP2D1 and CYP3A2 [Cleary et al. 1994; Chan et al., 2008]. Oxycodone metabolites are active and contribute significantly to its analgesic effect [Lalovic et al. 2004]. Since the outcome of treatments by codeine and oxycodone are intimately related to drug metabolism, these two drugs can be used to systemically study the influence of aging on CYP3A and CYP2D mediated drug metabolism.

A comprehensive understanding of the factors influencing drug metabolism are important for a safe and effective drug use in elderly patients. We therefore hypothesized that pharmacokinetic

alterations with aging can be largely due to drug metabolism impairment. We have recently generated pharmacokinetic and metabolism data on ketamine, a CYP3A substrate, to support this statement [Santamaria *et al.*, 2015]. The rate of drug metabolism by CYPs is amenable to modulation by structural changes in the enzymes [Sheweita, 2000]. Additionally, enzyme modifications can have a significant impact on the formation and elimination of active metabolites and consequently have a profound effect on the pharmacological and toxicological outcomes. Our recent study showed a significant CYP3A metabolic impairment in older rats leading to decrease in drug clearance [Santamaria *et al.* 2015; Giroux *et al.* 2016]. In geriatric patients this could therefore lead to numerous medical complications.

In the present study, functional changes will be characterized using two selective CYP2D and CYP3A substrates (i.e. codeine and oxycodone) and rat liver S9 fractions to define the metabolic stability and the enzyme-mediated clearance base on a Michaelis-Menten approach for specific metabolic pathways. Drug and metabolite analyses were performed using a HPLC-MS/MS method capable of quantifying codeine, norcodeine, morphine, oxycodone, oxymorphone and noroxycodone using an isotopic dilution strategy in liver S9 fraction suspensions.

Materials and Methods

Chemicals and Reagents

Codeine, d₃-Codeine, Norcodeine, d₃-Morcodeine, Morphine, d₃-Morphine, Oxycodone, d₃-Oxycodone, Noroxycodone, d₃-Noroxycodone, Oxymorphone and d₃-Oxymorphone were purchased from Cerilliant (Round Rock, TX). Other chemicals, including acetonitrile, formic acid, methanol, dibasic sodium phosphate and monobasic sodium phosphate, proteomic grade trypsin, dithiothreitol (DTT), iodoacetamide (IAA), hydrochloric acid (HCl), formic acid, LC-MS grade water, LC-MS grade acetonitrile, hexane, trifluoroacetic acid (TFA) were purchased

from Fisher Scientific (Ottawa, ON, Canada). Commercial rat liver S9 fractions and NADPH regeneration solutions were purchased from Corning Gentest (Tewksbury, MA, USA).

Animal study

Twenty-four specific pathogen free male Sprague Dawley rats from Charles River Canada (St-Constant, QC, Canada) were used for this study. Seven to nine weeks old rats (n=6/age group) were purchase and kept until they were respectively 3 and 6 months old. Twelve 8 month old rats were purchased and kept until they were 12 and 18 months of age (n=6/age group). All rats were housed in a standard laboratory animal environment. The rats had *ad libidum* access to food (2018 Teklad Global 18 % Protein Rodent Diet, Harlan Teklad, Bartonville, IL) and reverse osmosis water. They were single housed in ventilated cages (Green Line IVC Sealsafe Plus, Tecniplast, USA). Rats were housed on corn cob bedding (7097 corncob, Harlan Teklad, Bartonville, IL) which were changed once a week. They had a high temperature polycarbonate rat retreat (Bioserv, Flemington, NJ) and one Nylabone chew (Bioserv, Flemington, NJ) for environmental enrichment. The Institutional Animal Care and Use Committee of the Ste-Justine Hospital Research Center approved the protocol prior to animal use in agreement with the guidelines of the Canadian Council on Animal Care 1993. Rats were euthanized with CO₂, livers were rapidly remove and S9 fractions were prepared.

Rat Liver S9 preparation and incubation

For each age group, three livers were pooled and homogenized in a 50 mM TRIS-HCl buffer, pH 7.4, containing 150 mM KCl and 2 mM EDTA at a ratio of 1:4 (w:v). The homogenates were centrifuged at 9,000 g for 20 minutes. The total amount of protein in each supernatant was determined using the standard Coomassie protein assay (Bradford). Additionally, relative quantification of CYP2D and CYP3A was performed using a bottom-up proteomic strategy [C. Gröer et al. 2014] and results shown no statistical differences between age groups (data not shown). Supernatant aliquots were kept at -80 °C until usage. The incubations were performed as

previously described [Lavoie *et al.*, 2013; Santamaria *et al.* 2014]. All incubations were performed minimally in triplicates. The incubations were performed in 1.5 mL microcentrifuge tubes and contained various concentrations ranging from 1 to 100 μM of codeine or 1 to 250 μM of oxycodone, 0.5 mg/mL of S9 fraction proteins diluted in 100 mM phosphate buffer, pH 7.4. One mL of S9 enzyme suspensions were fortified with 50 μL of NADPH-regenerating solution A and 10 μL of solution B (Corning BD Biosciences Cat. No. 451200) and preincubated at 37°C for 5 min prior to the addition of codeine or oxycodone. Immediately after fortification of codeine or oxycodone into the liver S9 suspension containing the NADPH-regeneration system, the sampling point for $t = 0$ was taken, and further sampling points were taken at 5, 15, 30, 45, 60, and 90 min for metabolic stability experiments (i.e. substrate concentration was 2 μM). For the determination of K_m and V_{max} , the concentration of morphine, norcodeine, oxymorphone and noroxycodone were determined after 30 min incubation. Fifty μL of samples were taken and mixed with 250 μL of the deuterated internal standard solution (1 μM of deuterated internal standards in acetonitrile) in microcentrifuge tubes. The samples were centrifuged at 12,000 g for 10 min and 200 μL of the supernatant was transferred into an injection vial for HPLC-MS/SRM analysis.

Quantitative Analytical Methods

The concentrations of codeine, morphine, norcodeine, oxycodone, oxymorphone and noroxycodone were determined using an HPLC-MS/SRM assay. Two μL samples were injected using a Thermo Scientific Accela HPLC System (San Jose, CA, USA) onto a Thermo Biobasic C18 100 x 1 mm column (5 μm) with flow rate of 75 $\mu\text{L}/\text{min}$. The mobile phase consisted of a mixture of methanol, 10 mM ammonium acetate at a ratio of 40:60, respectively. The Thermo Scientific linear ion trap mass spectrometer (Thermo LTQ-XL) was interfaced with the HPLC system using a pneumatic assisted electrospray ion source. The mass spectrometer was coupled with the HPLC system using a pneumatically assisted electrospray ion source (ESI). The sheath

gas was set to 15 units and the ESI electrode was set to 4000 V in positive mode. The capillary temperature was set at 300 °C, the capillary voltage to 22 V. Specific MS parameters are shown in Table 1. All scan events were acquired with a 100 msec maximum injection time. Drug, metabolites and corresponding deuterium-labeled molecule analogues were analyzed in scan mode MS/MS. The quantification was based on specific post-processing SRM extracted ion chromatograms and analyte concentrations were determined using the peak area ratio of the light and heavy analogs. The analytical range used was ranging from 10 nM to 10 000 nM for all analytes. The method precision and accuracy were evaluated by analyzing twelve replicates of liver S9 enzyme suspension samples fortified with codeine, morphine, norcodeine, oxycodone, oxymorphone, noroxycodone at the nominal concentration of 10, 500 and 10 000 nM. Please note that NADPH solution was added after the protein precipitation to avoid degradation.

Data analysis and regression

All non-linear regression analyses were performed with PRISM version 6.0h (GraphPad, La Jolla, CA) using the non-linear curve-fitting module with an estimation of the goodness of fit. The Michaelis-Menten equation describes the rates of irreversible enzymatic reactions that are generally observed for CYP mediated metabolic reactions. Michaelis-Menten parameters can be estimated with non-linear regression analysis using the Michaelis-Menten equation [Michaelis and Menten, 1913].

$$v_i = \frac{v_{max} [S]}{K_m + [S]} \quad (1)$$

Where the initial velocity (v_i) was determined using equation 2.

$$v_i = \frac{d[P]}{dt} = \frac{[\text{morphine}]_{30 \text{ min}}}{30 \text{ min}} \text{ or } \frac{[\text{codeine}]_{30 \text{ min}}}{30 \text{ min}} \text{ or } \frac{[\text{oxymorphone}]_{30 \text{ min}}}{30 \text{ min}} \text{ or } \frac{[\text{noroxycodone}]_{30 \text{ min}}}{30 \text{ min}} \quad (2)$$

The initial rate (v_i) was calculated based on the metabolite concentrations measured after 10 minutes incubation of codeine or oxycodone in rat liver S9 enzyme suspensions. Following sample analysis, the analyte/deuterated internal standard peak area ratio was used to determine

drug and metabolite concentrations at various time points to determine the metabolic stability. All depletion data were fitted to the monoexponential decay model described in equation (3) where $C(t)$ are substrate concentration at time t and C_0 is the initial concentration.

$$C(t) = C_0 e^{-kt} \quad (3)$$

In vitro half-life was obtained using equation (4):

$$T_{1/2} = \frac{\ln(2)}{k} \quad (4)$$

Statistical analysis

Results are presented as means (\pm SD). The statistical difference was assessed with a one-way ANOVA and a Tukey's multiple comparisons test using PRISM software whereby; $p < 0.05$ was considered significant.

Results and Discussion

Tandem Mass Spectrometry

Precursor ion and product ion mass spectra for codeine, morphine, norcodeine, oxycodone, oxymorphone, noroxycodone and deuterated analog internal standards were obtained in positive ion mode. The precursor ion spectra of codeine, norcodeine, morphine, oxycodone, oxymorphone, noroxycodone showed an intense signal for the protonated molecule ($[M+H]^+$) at m/z 300, m/z 286, m/z 286, m/z 316, m/z 302 and m/z 302, respectively. Precursor ions detected for deuterated analogues were compatible with expected exact mass. The product ion spectra (MS^2) of codeine, morphine, norcodeine, oxycodone, oxymorphone, noroxycodone has predominant fragment ions at m/z 215, m/z 201, m/z 215, m/z 256, m/z 242 and m/z 229 respectively, as shown in Fig 2. As illustrated in Fig 3, the product ion spectra obtained were compatible with the molecular structures. The deuterated analog internal standards showed intense compatible fragment ions. The post acquisition SRM mass transition used are presented in Table 1 was set for best sensitivity and selectivity.

Calibration Curve Analysis

A linear regression (weighted 1/concentration) produced the best fit for the concentration–detector relationship. The regression model used was determined using the sum of the squares of the deviations [Beaudry, 1999]. By convention, the regression line is considered to properly fit the calibration set when the sum of squares of the deviations is minimized. The calculated coefficient of determination (R^2) were better than 0.990 for an analytical range set from 10 to 10 000 nM in liver S9 enzyme suspensions.

Precision and Accuracy

The reproducibility of the method was evaluated by analyzing twelve replicates of liver S9 enzyme suspension samples fortified with codeine, morphine, norcodeine, oxycodone, oxymorphone, noroxycodone at the nominal concentration of 10, 500 and 10 000 nM. The precision and accuracy results are displayed in Tables 2 and 3. The results obtained demonstrate that acceptable precision and accuracy results were achieved and were compatible with generally accepted criteria in bioanalysis (CDER and CVM, 2001). A representative chromatogram of LOQ samples is shown in Figure 4 and extracted blank rat liver S9 fraction did not show any interference from endogenous substances at the mass transition monitored for each analyte. Moreover, the signal-to-noise ratio of the LOQ sample was above 10:1 with a significant number of points to adequately determine peak area allowing the quantification of these analytes with acceptable figure of merits for a metabolism study.

Determination of the apparent Michaelis constant K_m and V_{max}

The rate of a specific metabolic pathway can be estimated using the Michaelis-Menten approach. The effect of substrate concentration on the initial rate (V_i) of an enzyme-catalyzed reaction is a central concept in enzyme kinetics. To adequately determine the value of the apparent Michaelis-Menten constant K_m as well as the maximum rate achieved by the system (V_{max}), the data were fitted with the Michaelis-Menten equation (1). The initial rate (V_i) was calculated using the

equation (2) and concentrations of morphine (CYP2D), norcodeine (CYP3A), oxymorphone (CYP2D) and noroxycodone (CYP3A) were determined after 30 min codeine or oxycodone incubation in rat liver S9 fraction suspensions by HPLC-MS/MS. Figures 5 and 6 show results coherent with kinetics following a Michaelis-Menten enzymatic reaction for all rat liver S9 fractions from all age groups and data were compatible with commercial rat liver S9 fractions (data not shown). The derived data are presented in Table 4. Codeine is a well-characterized substrate of CYP2D and the primary biotransformation product is morphine, the active compound. The observed K_m values were not significantly different when comparing age groups (Table 4 and Figure 5A). This is interesting because it suggests that with aging, the enzyme-substrate complex structure was not significantly different between codeine and rat CYP2D (i.e. CYP2D1). However, the derived V_{max} suggests a rapid saturation of the CYP2D enzyme active sites in liver S9 fractions of 18-month-old rats, thus affecting significantly the enzyme-mediated clearance ($CL_{U_{int}} = V_{max} / K_m$) of the drug. Norcodeine formation mediated by CYP3A was also monitored, but the observed initial rate of formation was very low and did not allowed to adequately estimate Michaelis-Menten parameters. No significant formation of norcodeine using rat liver S9 fractions or commercial rat liver S9 fractions was observed in this study, particularly at low substrate concentrations. Thus, we could not accurately determine K_m and V_{max} . Oxycodone is a known substrate of CYP2D and CYP3A leading to two specific metabolites, oxymorphone and noroxycodone respectively. As shown in Table 4 and Figure 5B/5C, the observed K_m values were not significantly different when comparing age groups despite some differences, which were noted (though not statistically significant) between 3-months-old rats compared with the other age groups. This observation is distinctive compared to rat liver CYP2D mediated metabolism of codeine. The K_m value is directly related to the thermodynamic stability of the binding cluster of oxycodone in the active site of CYP2D or CYP3A. The data suggests differences showing a smaller K_m values that may indicate that oxycodone may have stronger

interactions with CYP2D or CYP3A active site residues leading to more thermodynamically stable enzyme-substrate complexes. This may suggest drug metabolism changes with aging, a phenomenon observed in special populations groups like pediatric patients [Bartelink et al. 2006]. The derived V_{\max} for CYP2D (oxymorphone) and CYP3A (noroxycodone) were compared and a rapid saturation of both enzyme active sites in liver S9 fractions of 18-month-old rats was observed, thus drastically affecting the enzyme-mediated clearance ($CL_{u_{int}}$) of oxycodone. The rates of formation of morphine, oxymorphone and noroxycodone undergo a very rapid saturation in the liver S9 fractions of 18-month-old rats suggesting that following a normal dose, drug metabolism can be impaired leading to a significant increase of drug exposition (AUC) and hampered elimination.

Codeine and oxycodone metabolic stability evaluations in rat liver S9 fractions

The *in vitro* metabolic stability (i.e $T_{1/2}$) and enzyme-mediated clearance (V_{\max} / K_m) were used to characterize functional modifications of CYP2D and CYP3A proteins with aging. The systemic drug exposition is intimately related with the metabolic stability. Codeine and oxycodone metabolic stability at 2 μM ($[S] < K_m$) was evaluated in all age groups of rat liver S9 fractions. The data presented in Figure 6A/6B were fitted with a mono-exponential decay model as described in equation (3) and the coefficients of determination (R^2) were comprised between 0.85 and 0.96 for each fitted data series, suggesting that codeine and oxycodone first-order kinetics were observed and the conditions of the model were met [Masimirembwa *et al.*, 2001]. The derived $T_{1/2}$ were significantly higher for experiments performed using 18-months-old liver S9 fractions compared to all other age groups (Table 4). These results are compatible with the enzyme-mediated clearances observed, and altogether suggest that systemic exposure will significantly increase with aging and drug clearance will be impaired leading to potentially undesired clinical outcomes.

Conclusion

Age and related factors have a substantial effect on codeine and oxycodone bioavailability that are related to physiological changes but also biochemical changes such as liver metabolism alterations. The quantification of CYP2D and CYP3A enzyme levels reveals no statistical difference between age groups. Thus, significant decreases in the rate of formation of specific metabolites could be related to rat CYP2D and CYP3A conformational changes with age particularly in the geriatric group (18-month-old rats). More specifically, the results strongly suggest a severe impairment of CYP2D codeine metabolism as well as CYP2D and CYP3A oxycodone mediated metabolism. Almost 50% of marketed drugs were metabolized by CYP3A and another 30% by CYP2D. Consequently, functional modifications of CYP enzymes can lead to severe consequences including alterations in efficacy, safety and toxicity of these drugs. Moreover, functional modifications of CYP enzymes may exacerbate problems associated with drug-drug interactions. Older patients under opioid treatments are most of the time considered medically complicated patients, whom might be taking multiple medications and may suffer from inflammation as well as renal and hepatic impairments. They are therefore more susceptible to potentially harmful drug-drug interactions. It is therefore essential to improve our understanding of the fundamental mechanisms associated with metabolic and pharmacological changes that occur with aging in order to develop predictive methods to aid the optimization of drug administration, to obtain the desired efficacy and to reduce or eliminate undesired clinical outcomes.

Acknowledgments

This project was funded by the National Sciences and Engineering Research Council of Canada (F.Beaudry discovery grants no. 386637-2010 and RGPIN-2015-05071). The HPLC-MS/MS analyses were performed on instruments funded by the National Sciences and Engineering

Research Council of Canada (F.Beaudry Research Tools and Instruments Grants no. 439748-2013).

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Table 1. Summary of compound specific parameters used for HPLC-MS/MS analysis.

Compound	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Collision Energy (%)
Codeine	300.2	215.1	45
d ₃ -Codeine	303.2	215.1	45
Morphine	286.2	201.1	45
d ₃ -Morphine	289.2	201.1	45
Norcodeine	286.2	215.1	40
d ₃ -Norcodeine	289.2	218.1	40
Oxycodone	316.2	256.1	45
d ₃ -Oxycodone	319.2	259.1	45
Oxymorphone	302.1	242.1	45
d ₃ -Oxymorphone	305.1	245.1	45
Noroxycodone	302.1	229.1	40
d ₃ -Noroxycodone	305.1	232.1	40

Table 2. Precision and accuracy evaluation for codeine and phase 1 metabolites in supernatant of Rat liver S9 fractions

Compound	Concentration (nM)	Mean (n = 12)	RSD (%)	RE (%)
Codeine	10	9.36	3.0	-6.4
	500	569.4	2.6	12.3
	10000	9247.4	2.7	-7.5
Morphine	10	9.62	12.7	-3.8
	500	539.7	2.7	7.9
	10000	9912.9	3.4	-0.9
Norcodeine	10	10.99	7.7	9.9
	500	532.8	10.4	6.6
	10000	10030.2	2.5	0.3

RSD = Relative Standard Deviation, RE = Relative Error

Table 3. Precision and accuracy evaluation for oxycodone and phase 1 metabolites in supernatant of rat liver S9 fractions

Compound	Concentration (nM)	Mean (n = 12)	RSD (%)	RE (%)
Oxycodone	10	9.09	4.4	-9.1
	500	462.9	8.1	-7.4
	10000	9485.5	5.0	-5.1
Oxymorphone	10	10.37	8.0	3.7
	500	530.5	11.5	6.1
	10000	9241.1	6.1	-7.6
Noroxycodone	10	9.12	6.4	-8.8
	500	504.6	10.6	0.9
	10000	9194.5	8.7	-8.1

RSD = Relative Standard Deviation, RE = Relative Error

Table 4. Kinetic parameters associated with the metabolism of Codeine and Oxycodone in rat liver S9 fractions

	$V_{\max} \pm \text{SD}$ $\text{nmol min}^{-1} \text{mg}^{-1}$	$K_m \pm \text{SD}$ μM (nmol mL^{-1})	$T_{1/2} \pm \text{SD}$ min
Codeine			
3 months			126.1 ± 17.7
Morphine (CYP2D)	0.327 ± 0.027	10.3 ± 3.4	
6 months			101.7 ± 14.4
Morphine (CYP2D)	0.355 ± 0.021	9.0 ± 2.2	
12 months			162.6 ± 32.8
Morphine (CYP2D)	0.273 ± 0.028 ^a	11.4 ± 4.4	
18 months			225.2 ± 54.2 ¹
Morphine (CYP2D)	0.092 ± 0.003 ^b	10.9 ± 1.2	
Oxycodone			
3 months			180.6 ± 16.9
Oxymorphone (CYP2D)	0.273 ± 0.033 ^c	33.5 ± 12.2	
Noroxycodone (CYP3A)	1.13 ± 0.17 ^c	47.6 ± 19.4	
6 months			170.5 ± 32.7
Oxymorphone (CYP2D)	0.690 ± 0.096	60.7 ± 21.1	
Noroxycodone (CYP3A)	2.92 ± 0.47	61.1 ± 24.7	
12 months			178.1 ± 13.9
Oxymorphone (CYP2D)	0.499 ± 0.087 ^d	42.8 ± 20.9	
Noroxycodone (CYP3A)	2.00 ± 0.34 ^d	49.5 ± 22.5	
18 months			270.7 ± 39.1 ²
Oxymorphone (CYP2D)	0.153 ± 0.010 ^e	42.8 ± 7.8	
Noroxycodone (CYP3A)	0.409 ± 0.053 ^e	40.6 ± 14.8	

^a $p < 0.01$ compared to 6 months

^b $p < 0.0001$ compared to 3, 6 and 12 months

^c $p < 0.001$ compared to 6 months; $p < 0.05$ compared to 12 and 18 months

^d $p < 0.05$ compared to 6 months

^e $p < 0.0001$ compared to 6 and 12 months

¹ $p < 0.05$ compared to 3 months; $p < 0.01$ compared to 6 months

² $p < 0.05$ compared to 3 months and 12 months; $p < 0.01$ compared to 6 months

List of Figures

Figure 1. Specific Phase 1 Liver CYPs mediated metabolism of codeine (A) and oxycodone (B)

Figure 2. Product ion spectra (MS^2) for codeine, oxycodone and their respective major CYPs metabolites. A) MS^2 spectrum of codeine; B) MS^2 spectrum of morphine; C) MS^2 spectrum of norcodeine; D) MS^2 spectrum of oxycodone; E) MS^2 spectrum of oxymorphone; F) MS^2 spectrum of noroxycodone

Figure 3. Proposed MS^2 fragmentation mechanism. A) MS^2 principal product ions for codeine; B) MS^2 principal product ions for morphine; C) MS^2 principal product ions for norcodeine; D) MS^2 principal product ions for oxycodone; E) MS^2 principal product ions for oxymorphone; F) MS^2 principal product ions for noroxycodone

Figure 4. Overlay reconstructed ion chromatograms of blank, LOQ and a reference samples for codeine, morphine, norcodeine, oxycodone, oxymorphone and noroxycodone.

Figure 5. Determination of Michaelis constant K_m and maximum velocity V_{max} using non-linear regression fitting. The initial rate of formation (V_i) was measured for morphine (A), Oxymorphone (B) and Noroxycodone (C). Each point represents the mean (\pm SD) of experiments in triplicate. Significant differences of the initial rate of formation (V_i) were observed starting at 2 μ M substrate concentration for liver S9 fractions of 18-month-old rats.

Figure 6. Depletion profile of codeine (A) and oxycodone (B) in rat liver S9 fractions rat at 2 μ M. Data presented on a linear scale and each point represents the mean (\pm SD) of triplicate experiments. The half-life of both drugs is significantly increased in 18-month-old rat liver S9 fraction.

Figure 1.

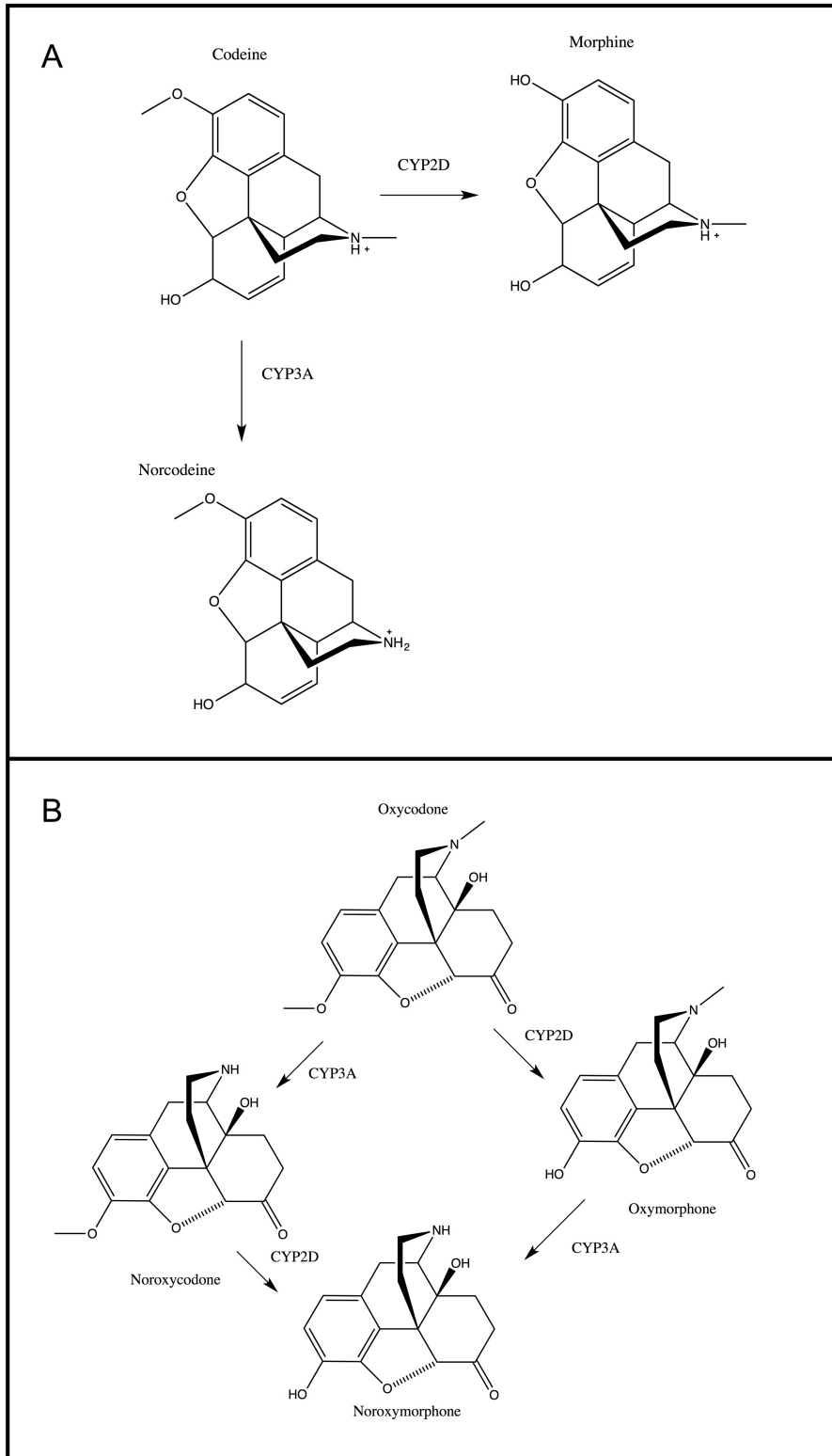


Figure 2.

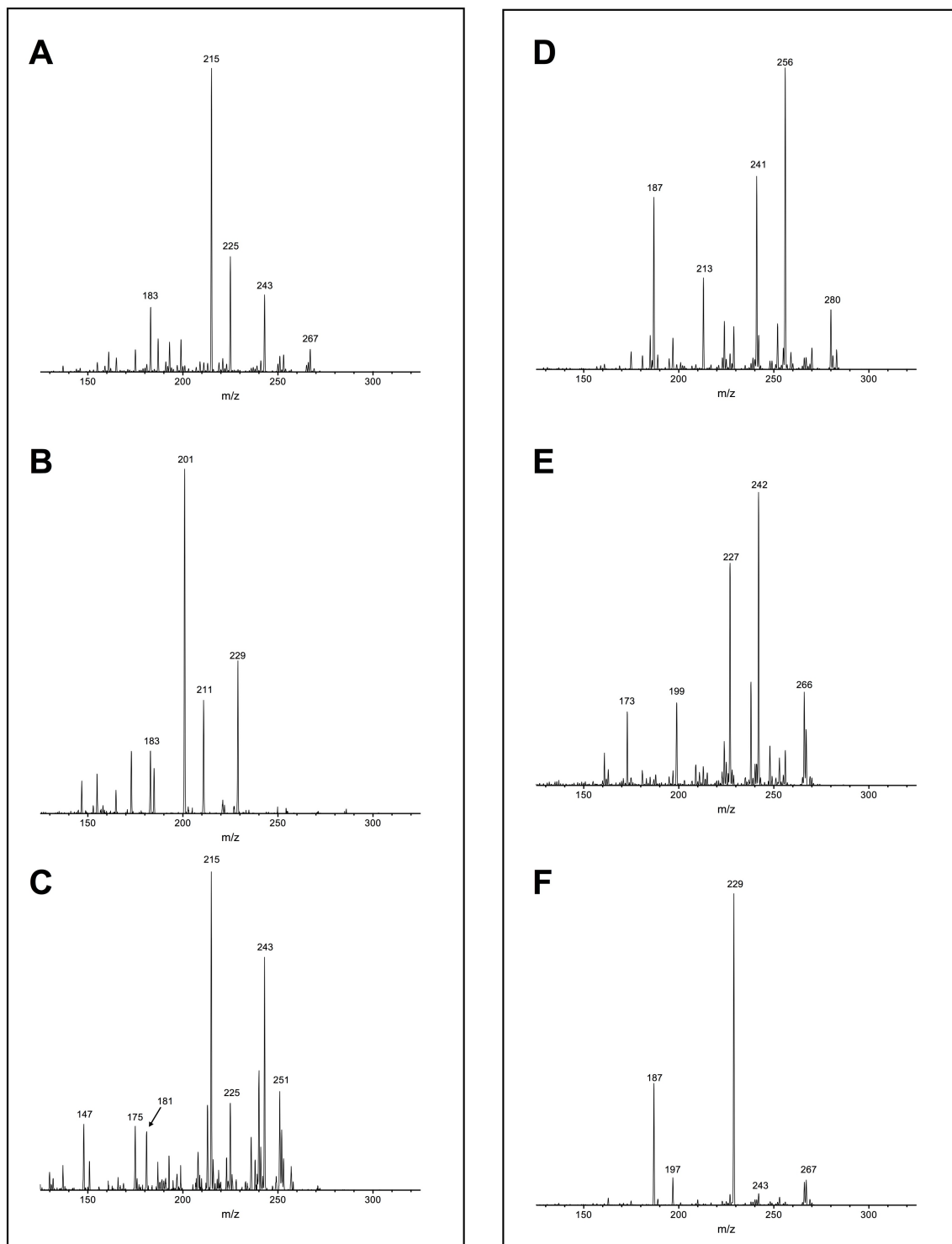


Figure 3.

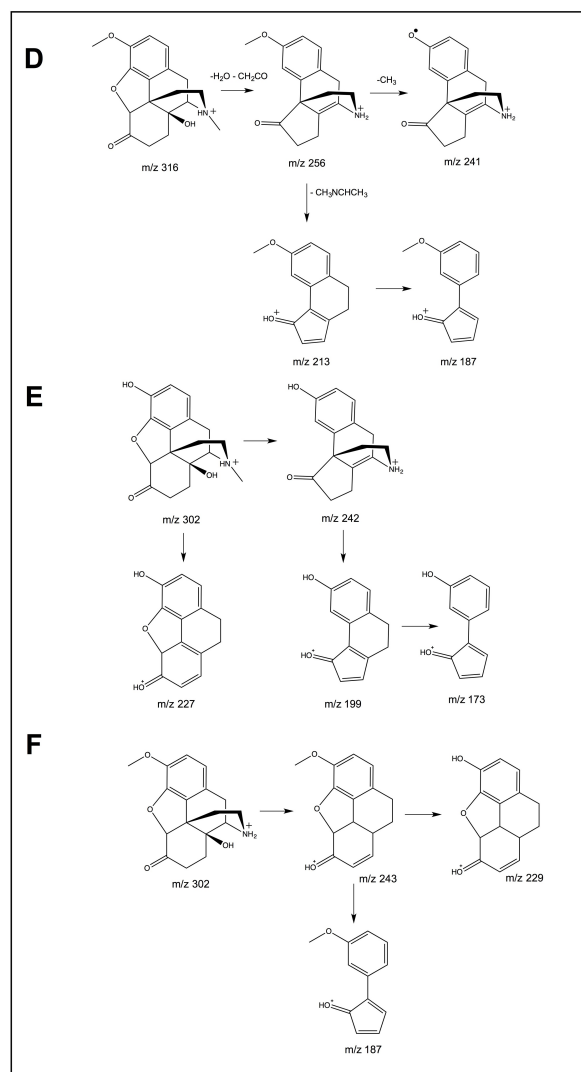
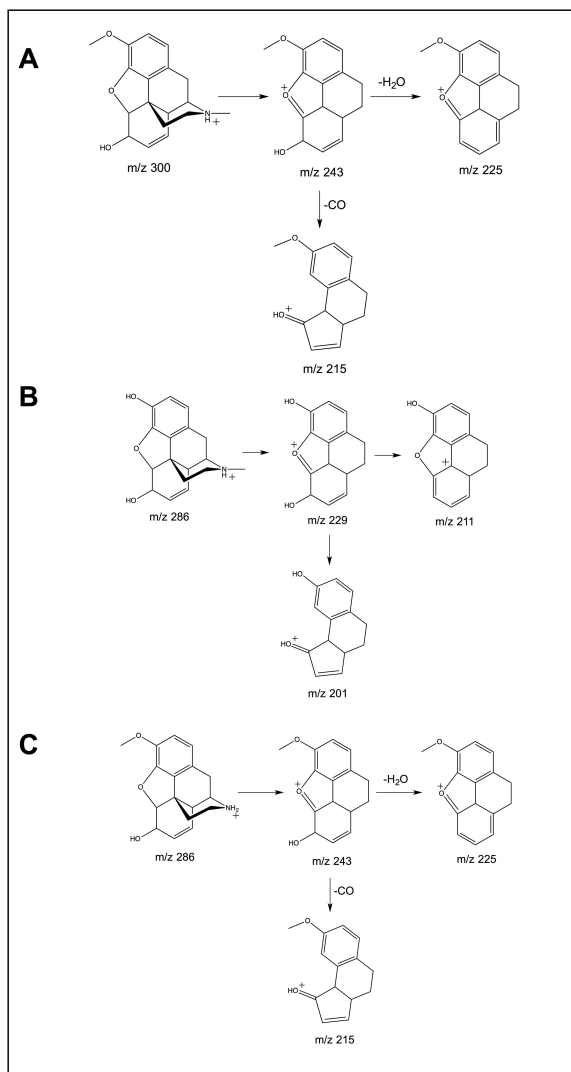


Figure 4.

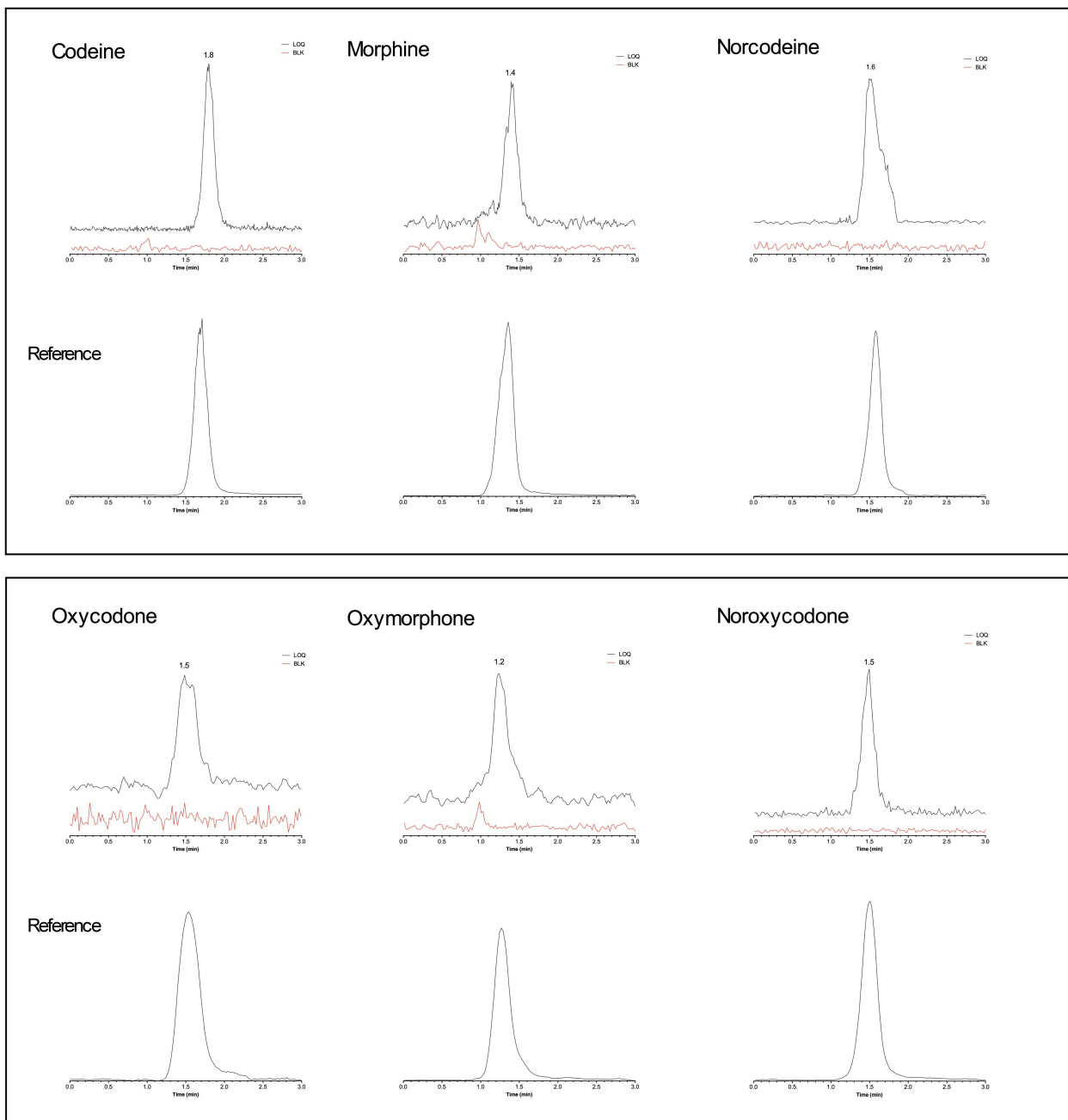
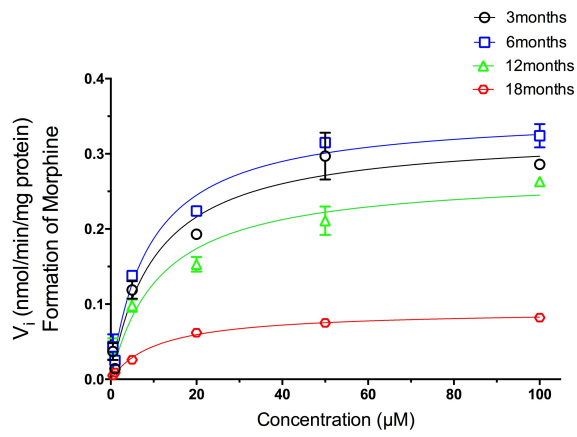
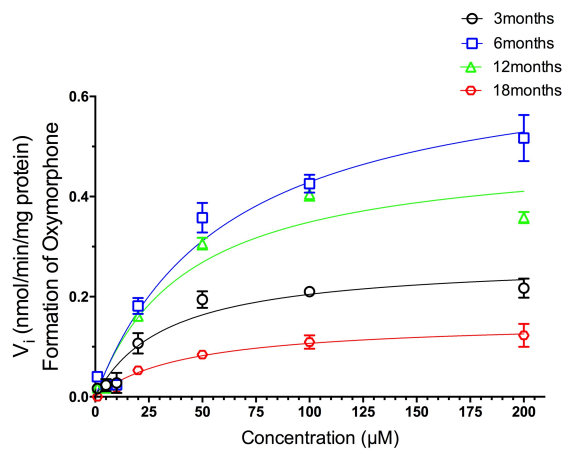


Figure 5.

A



B



C

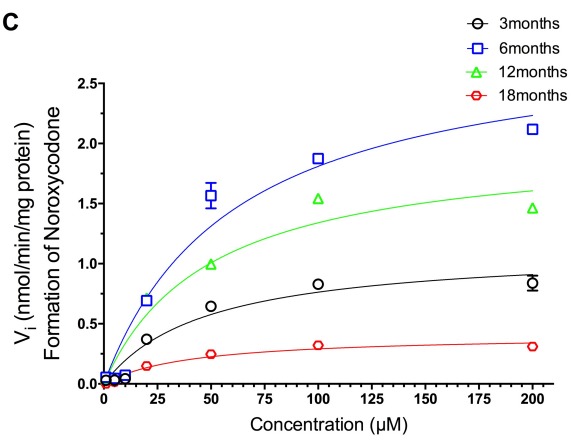
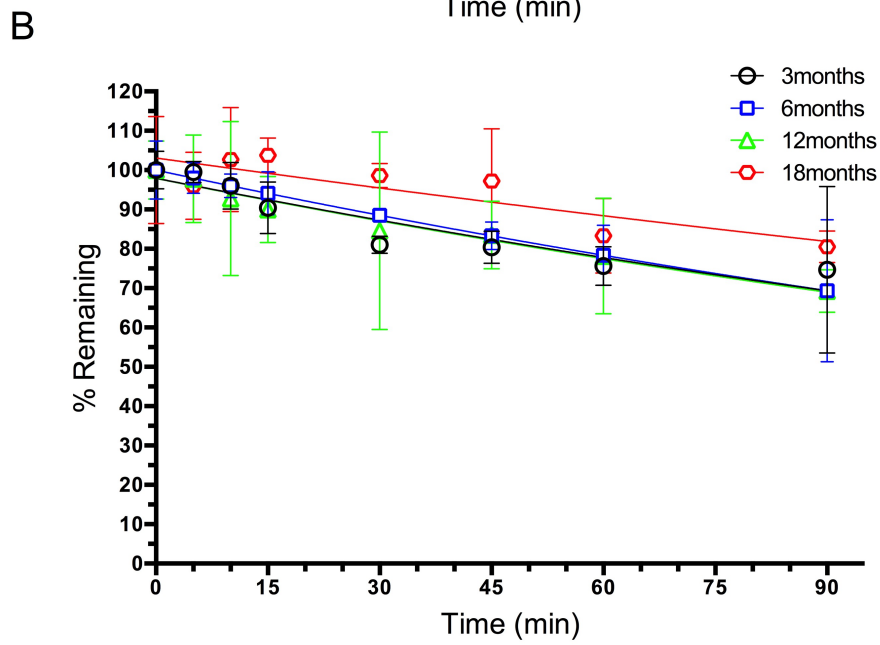
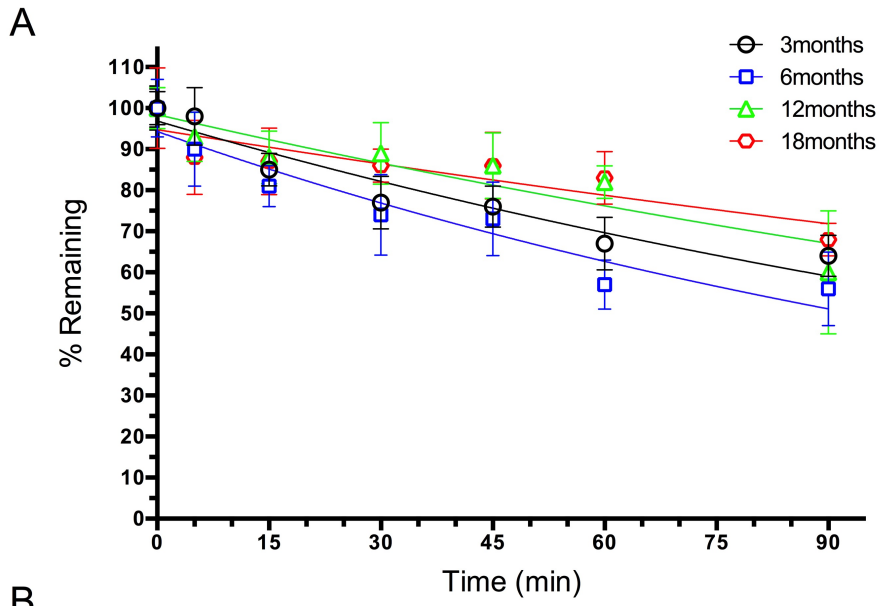


Figure 6.

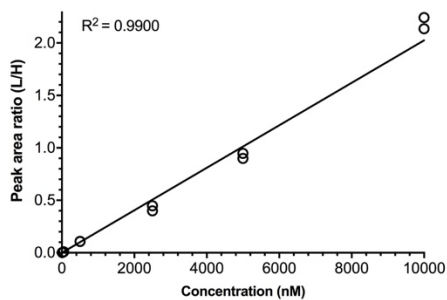


Supplemental figure

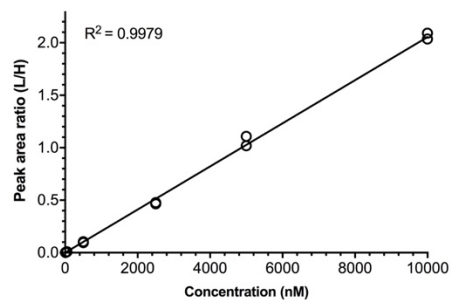
Legend

Figure S1. Examples of calibration curves for each targeted analyte. Rat liver S9 fractions were fortified at six nominal concentrations and drugs and metabolites were extracted. Peak area ratios of drugs and metabolites and corresponding deuterium labeled analogs were used to construct the calibration curve. Deuterium labeled analogs were used at a constant concentration of 1 μM . The observed coefficient of determination (R^2) was ≤ 0.99 .

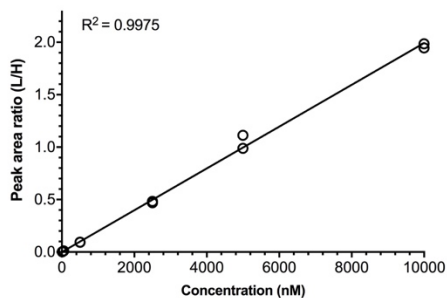
Codeine



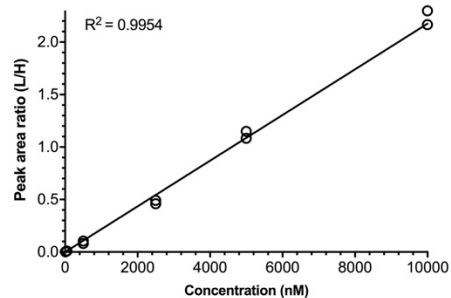
Oxycodone



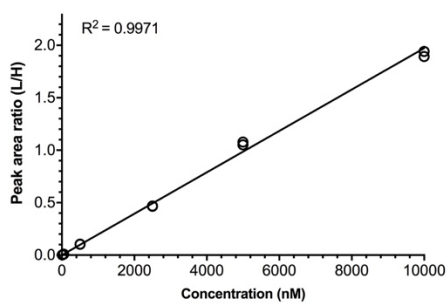
Morphine



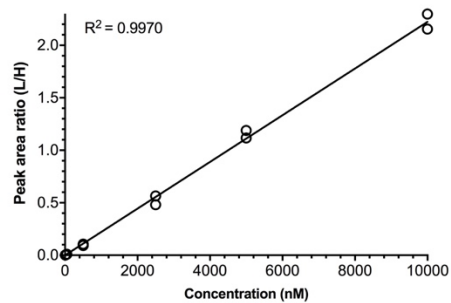
Oxymorphone



Norcodeine



Noroxycodone



IV Discussion

The evaluation of metabolism involved in the clearance of drugs is essential for the understanding of the cause of increased toxicity or decreased efficacy of drugs. In the literature, a decrease in clearance of drugs in geriatric patients has been reported (Mangoni & Jackson, 2004) and in common practise, the dose of drugs are generally decreased with age (Turnheim, 2003). This decrease has been attributed to physiological changes like the decrease in blood flow, reduced liver mass, decreased renal weight and number of glomeruli in the kidneys but the specific mechanisms involved in the decrease of metabolism of drugs by the liver (Mangoni & Jackson, 2004) is still being further investigated.

Opioids are an important class of drugs that are generally prescribed to reduce pain, but their toxicity can be severe. In clinical settings for elderly patients, opioids are generally titrated to reach an optimum dose and prevent the toxicity due to an overdose. Pharmacokinetic and pharmacodynamic studies regarding the effect of age on opioid clearance are scarce. Some of the existing theories include decreased liver perfusion, reduced liver mass and decreased lean body mass ratio. The CYP450 enzymes present in the liver are a major clearance pathway for a large percentage of pharmaceutical drugs currently prescribed in human and veterinary medicines. CYP2D and CYP3A are known to be responsible for the metabolism of a wide range of drugs including but not exclusive to analgesics, antidepressants and antipsychotics (Pinto & Dolan, 2011). Impairment or hinderance of these enzymes could lead to severe drug toxicity or inefficacy due to the accumulation of drugs in the the body.

CYP2D is an important liver enzyme responsible for metabolising opioids. Pharmacogenetic differences have resulted in different phenotyping of humans into PM, IM, EM and UM. Codeine, a prodrug activated by this enzyme, has caused a large controversy where pharmacodynamic responses ranged from respiratory depression due to morphine toxicity and complete absence of analgesia. On a closer reexamination of the cases reported, toxicity due to morphine accumulation were always in children whereas the cases reporting absence of analgesia were at the geriatric age. The severe side effects of codeine enabled a clear observation of the trend of decreasing liver metabolism with aging in the clinical setting, but for other drugs that follow the CYP2D metabolism pathway, accumulation of the drugs causing only minor discomfort or milder side effects can leave them unnoticed.

Furthermore, a study of rectal codeine administration for post-operative analgesia in infants and children aged between 6 months and 4 years reported the mean initial half-life was 2.6 hours, but in the infants of the lower body weight, the half-life was over 2 hours longer (Quiding *et al.*, 1992). Another study reported that the plasma drug concentration indicated that a rectal dose of codeine of 0.5 mg/kg in children can result in similar, or slightly greater, plasma concentrations of codeine and its metabolites than after a 60 mg oral dose in adults (Quiding *et al.*, 1986). Furthermore, a single-dose analysis of codeine offered no superior effect to placebo effect in adults following surgery or child birth (Honig & Murray, 1984). No significant advantage of codeine over placebo was observed in older patients with cancer pain but constipation as a side effect of codeine was observed (Jochimsen & Noyes, 1978). On the basis of available research findings, age specific differences in the pharmacokinetics of codeine may be significant (McEwan *et al.*, 2000). In rodent model the evaluation of CYP3A substrate clearance have shown a consistent decrease with aging but this has not been observed in humans. No decrease in CYP3A activity or content with age was shown in human liver microsomes (Parkinson *et al.*, 2004; Schmucker *et al.*, 1990). Recently, a reduced metabolism of ketamine in geriatric rats was reported (Santamaria R. *et al.*, 2015.)

Summary of results

The experiments performed in this project were focused on the evaluation of CYP2D and CYP3A metabolic function with age using rat liver S9 fractions. Codeine and oxycodone were selected for use to evaluate the metabolic stability of CYP2D and CYP3A because they are well characterised substrates. The designed HPLC-MS method, allowed for selective quantification of codeine and its metabolites morphine and norcodeine and oxycodone and its metabolites noroxycodone and oxymorphone. The amount of noroxycodone could not be detected using the HPLC-MS method which might be due to low concentrations of the metabolite formed. The CYP2D and CYP3A content in relation to age, were quantified using bottom up proteomics (Groer *et al.*, 2014). No significant difference in CYP2D and CYP3A content between different age groups were found. This is consistent with a previous publication (Schmucker *et al.*, 1990) in which the authors reported that Phase I enzymes did not show change in CYP450 content with age.

The data generated by the HPLC-MS was fit into Michaelis-Menten equation. The presence of metabolites; morphine for codeine, oxymorphone and noroxycodone for oxycodone, were statistically compared between the different age groups. Results generated showed a clear decrease in the formation of morphine, oxymorphone and noroxycodone at the geriatric age. For codeine, the observed K_m for morphine (CYP2D) values were not significantly different when comparing age groups. However, the derived V_{max} suggests a rapid saturation of the CYP2D enzyme active site in 18-month old rats, thus affecting the enzyme mediated clearance of codeine. For oxycodone, the observed K_m values for oxymorphone (CYP2D) and noroxycodone (CYP3A) showed no significant difference between different age groups but some differences, not statistically significant were noted which were not observed with codeine. These differences might be attributed to the thermodynamic stability of the binding cluster of oxycodone to the active site of CYP2D or CYP3A. The data suggests that oxycodone may have a stronger interaction with CYP2D than CYP3A active site residues leading to more thermodynamically stable enzyme-substrate complex. Surprisingly for oxymorphone and noroxycodone, low concentrations were seen at three month-old rats, but if the K_m and V_{max} values are used to evaluate the clearance between different age groups, we might observe that at three months, the low value of V_{max} might be compensated with the K_m value. The results show a significant impairment in the metabolism of opioids by CYP2D and CYP3A in geriatric rats.

On a close observation, a pattern is seen where initially the metabolism by the two specified enzymes is low, it attains its maximum by middle age, then starts decreasing until finally it is impaired at the geriatric age. It is thus interpreted here that the enzymes develop till they attain enzymatic maturity and then start losing functionality after a certain age. This observation is consistent with the clinical cases reported in humans and the results obtained can be used to interpret the difference in pharmacodynamic response for different age groups observed in humans.

The elimination half life was significantly higher for liver S9 fractions from the 18-month old rats compared to all other age groups for both codeine and oxycodone. The results were compatible with the observed enzyme-mediated clearance and when combined, it suggests that systemic exposure to codeine and oxycodone will significantly increase with age and drug clearance will be impaired leading to potential undesirable outcomes.

Relevance and Implications of observations

According to the proposed hypothesis for the present study, the results clearly show impairment in CYP2D and CYP3A metabolism activity with age. This impairment could lead to the accumulation of drugs in the body. In the case of codeine, its accumulation could lead to side effects, such as itching, nausea, vomiting, coughing and constipation. Though they are not severe side effects, taking into consideration that codeine is often prescribed to geriatric patients to relieve mild to moderate pain, this will lead to absence of relief from pain in addition to the feeling of discomfort due to side effects. Elderly patients are also known to have bowel problems like constipation and codeine accumulation can worsen this in the elderly. Furthermore, pain that is not effectively treated may develop into chronic pain and hence elderly patients on codeine treatment may be at a higher risk of developing chronic pain. Even with the recent advancement in medicine, chronic pain still has no reliable treatment or cure and it is best to treat pain at its early stages before it develops into chronic pain. In the case of oxycodone, impairment of enzymatic clearance of the drugs could lead to severe side effects due to drug accumulation. The side effects of oxycodone include pinpoint pupils, constipation, nausea, vomiting, weak pulse, low blood pressure and respiratory depression leading to coma and death due to opioid overdose.

The clinical observations related to codeine have led to the proposal of age altered metabolism. Earlier studies in humans for CYP1A activity showed a decrease with age (Schwartz, 2007). CYP3A impairment in geriatric rats has also been reported with other drugs (Cotreau *et al.*, 2005; Giroux M.C. *et al.*, 2015; Patki *et al.*, 2004; Schmucker, 2001; Santamaria R. *et al.* 2015). CYP2C9 and CYP2C19 showed a decrease in a clearance in the order of 25%, and that elderly extensive metabolizers were phenotypically closer to elderly poor metabolizers than the young extensive metabolizers to the young poor metabolizers (Ishizawa *et al.*, 2005). Combining the previous data with CYP2C9 and the data obtained for this experiment, an assumption that with age, extensive metabolizers might become poor metabolizers necessitating the need to decrease the dose of drugs metabolized by CYP2D and CYP3A in geriatric patients. Prodrugs that are activated by CYP2D and CYP3A enzymes might have to be revised to ensure patients are not being given drugs that are not activated, leading to aggravations of their symptoms and the worsening of already existing diseases.

Functional modification of CYP2D and CYP3A enzymes can lead to severe consequences including alterations in efficacy, safety and toxicity of drugs. In elderly patients this is further

complicated by the presence of diseases like hepatic impairment and renal failure (Tajiri K. & Shimizu Y., 2013). Drug-drug interactions in this population due to polypharmacy also adds another problem to an already complex situation. The presence of drugs that can induce or inhibit CYP3A and CYP2D activity adds a new dimension to the complexity of the situation. The understanding of the exact mechanism associated with metabolic, psychological and pharmacological changes that occur with age is essential for the understanding, proper development and optimization of drug regimens in elderly patients. This will result in drug regimens that can produce the desired efficacy while reducing or eliminating undesirable side effects.

CYP3A and CYP2D metabolism was found to be impaired in geriatric rats but comparing our findings with previously published data on CYP3A impairment in geriatric animals, that could not be interpreted into human, where a decrease in human CYP3A metabolism was not clinically observed, the results of this project will have to be carefully interpreted to clinical studies and further investigated. The impairment of CYP2D and CYP3A with age can be interpreted in terms of post translational modification in protein structure and misfolding, which can be correlated with an accumulation of oxidative stress damage. This will require further investigation through molecular modelling .

Limitations

Previous studies have shown that bacterial lipopolysaccharides induce the release of intermediary cytokines, which in turn induce nitric oxide (NO) activity in Kupffer cells and hepatocytes (Khatsenko *et al.*, 1993). NO is known to bind to both the ferric and the ferrous hemoproteins in intact CYP450 and prevents oxygen binding, thereby blocking enzyme activity. The capacity of NO to bind to CYP450 indicates that the decrease in total CYP450 could reflect masking of CO binding by NO resulting in the inactivation of CYP rather than a true decrease in CYP450 content. NO may also enhance degradation of CYP450 by nitrosylation of heme or thiols in CYP450 apoprotein or impair transcriptional activation of CYP450. NO-mediated suppression of CYP450 could have an important impact on the pharmacotherapy of patients who have infection or are undergoing cancer chemotherapy with cytokines (Khatsenko *et al.*, 1993).

It has also been reported that high fat diet alters intestinal microbial profile and increases intestinal permeability, leading to increased production and leaking of lipopolysaccharides (Zhao

& Chen, 2015). Lipopolysaccharides levels were also found to be elevated in the circulation of aged humans. Obese patients have also been shown to have increased levels of lipopolysaccharides. Also results from one study show that an acute injection of lipopolysaccharides induce the gene expression of cytokines (Zhao & Chen, 2015). This information combined with the NO binding to CYP40 reducing its activity adds another limitation to this project which is the consideration of obesity and effect of aging on fat metabolism and lipopolysaccharides.

Finally, recent studies have shown that non-alcoholic fatty liver diseases decrease *in vivo* CYP3A activity. Furthermore liver fibrosis was also associated with a decrease in CYP3A enzyme function. A reduction in the expression of CYP3A mRNA *in vivo* was reported in non-alcoholic fatty liver diseases when compared to healthy subjects (Woolsey *et al.*, 2015). The effect of liver diseases on CYP2D and CYP3A function should also be taken into consideration to broaden our knowledge about the reasons for the impairment of these two enzymes in the geriatric age.

V Conclusion

The *in vitro* experiments performed in the present study using rat liver S9 fractions from different age groups demonstrated that CYP2D and CYP3A metabolism are severely impaired with age. Since 50% of drugs are metabolized by CYP3A and another 30% by CYP2D the efficacy of drugs bioactivated by these two enzymes might decrease with age. This can lead to parent drug accumulation causing undesirable side effects without any drug efficacy. As for drugs that are metabolised to be cleared through CYP2D and CYP3A, the drugs might accumulate leading to toxicity due to high concentration of drug in the body. The accumulation of xenobiotics in the body is known to cause diseases such as organ failures, respiratory depression, coma which can ultimately lead to death. As such the dose of drugs metabolized by these enzymes might have to be decreased with age and optimization of drug has to be performed. In the case of CYP2D, phenotyping of patients might be more relevant at the geriatric age than genotyping as seen in the case of CYP2C1. The use of codeine as a mild analgesic might be questionable since impaired CYP2D activity means diminished morphine formation. Previous studies have shown that PM suffered from side effects of codeine without experiencing any pain relief.

Animals, especially dogs, are at a greater risk for developing harmful effects from the impairment of these two enzymes because of the presence of various races from the same species which makes it harder to predict the phenotype in each race. Polymorphism in different races of companion animals is not well studied and hence the effect of age on CYP2D for each race is hard to determine. This emphasizes the need to classify and study companion animals and their respective genotype and phenotype further for a better understanding of metabolism and to enhance the efficacy and decrease the toxicity of drugs being prescribed. Companion animals at geriatric age are known to suffer from similar diseases like geriatric humans including arthritis, diabetes and cancer. They are prescribed opioids for enhancement of the quality of life. Careful monitoring of opioids has to be done to prevent side effects and toxicity which is the result of impaired CYP2D and CYP3A metabolism. Cats are known to be deficient in Phase II metabolism, which can make geriatric cats more susceptible to the impairment of these enzymes since they lack an alternative pathway for the clearance of drugs.

Although further studies are required to establish the exact mechanism by which CYP2D and CYP3A lose their functionality with age, the findings of our project suggest a severe impairment of metabolism which is a first step towards adjusting the doses of drugs in elderly patients including animals to enhance efficacy, maintain quality of life, decrease toxicity and prevent development of chronic pain. The results also highlight the importance of developing analgesics and other classes of drugs that are not metabolized by these enzymes to prevent complications in elderly patients. In addition to that, it clearly defines the importance of phenotyping rather than genotyping patients at different age groups for proper drug optimization.

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VII Annex

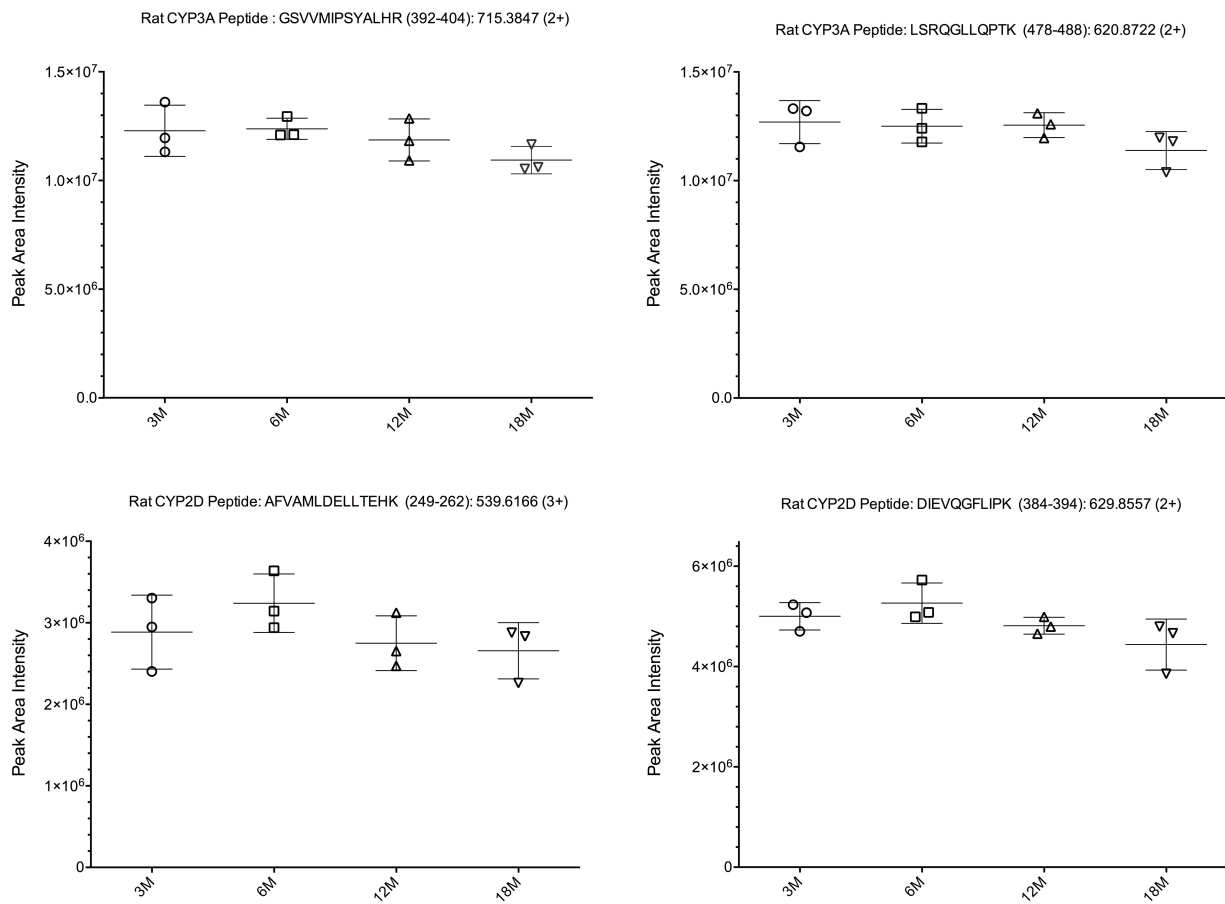


Figure 9: The content of CYP2D and CYP3A after bottom-up proteomics showed no statistical difference between different age groups when statically compared using ANOVA and Tukey's multiple comparison test.

Bottom-up proteomics refers to the characterization of proteins by analysis of peptides released from the protein through proteolysis. It provides an indirect measurement of proteins through peptides derived from proteolytic digestion of intact proteins. The peptide is fractionated and subjected to LC-MS/MS analysis. In this study liver S9 fractions were digested with trypsin and analyzed with a high-resolution Orbitrap MS.