

2010

## **Role of gastrointestinal multidrug resistance (MDR1) gene and P-glycoprotein (P-gp) in the oral absorption of methadone in horses**

Renata Lehn Linardi

*Louisiana State University and Agricultural and Mechanical College*

Follow this and additional works at: [https://digitalcommons.lsu.edu/gradschool\\_dissertations](https://digitalcommons.lsu.edu/gradschool_dissertations)



Part of the [Veterinary Medicine Commons](#)

---

### **Recommended Citation**

Linardi, Renata Lehn, "Role of gastrointestinal multidrug resistance (MDR1) gene and P-glycoprotein (P-gp) in the oral absorption of methadone in horses" (2010). *LSU Doctoral Dissertations*. 2938.  
[https://digitalcommons.lsu.edu/gradschool\\_dissertations/2938](https://digitalcommons.lsu.edu/gradschool_dissertations/2938)

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact [gradetd@lsu.edu](mailto:gradetd@lsu.edu).

ROLE OF GASTROINTESTINAL MULTIDRUG RESISTANCE (MDR1) GENE AND  
P-GLYCOPROTEIN (P-gp) IN THE ORAL ABSORPTION OF METHADONE IN HORSES

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The Department of Veterinary Medical Sciences

by

Renata Lehn Linardi

D.V.M., University of Sao Paulo State - UNESP, Brazil 2000

M.S., University of Sao Paulo State - UNESP, Brazil 2004

May 2010

## **DEDICATION**

To my loved family, my parents Marcos A. N. Linardi and Beatriz H. Linardi and my siblings Lizandra, Adriana and Juliana L. Linardi for unconditional support during this journey, more than ever.

To my husband Andrew D. Augustine for his friendship, complicity and love.

To the Horse:

“Sempre minha inspiracao para tornar meus sonhos realidade”

Always an inspiration to make my dreams come true.

## **ACKNOWLEDGEMENTS**

I would like to thank Dr. Claudio Natalini who gave me the first opportunity toward this degree and Dr. Rustin Moore who encouraged me to pursue my goals.

I am especially grateful to Dr. Ashley Stokes for the guidance as my advisor and friendship during these years at LSU. I am also thankful to the members of my committee Dr. Konstantin Gus Kousoulas, Dr. Frank M. Andrews, and Dr. Inder Sehgal for their support and contribution to my research.

I would like to thank Dr. Charles Short for his kindness, friendship, and scientific contribution to this dissertation.

I thank the faculty and staff of the Veterinary Clinical Sciences (VCS), Pathobiological Sciences (PBS), and Comparative Biomedical Sciences (CBS) departments at the School of Veterinary Medicine at LSU for providing the material and intellectual means necessary for the completion of this research. I want to thank especially Mr. Michael Kearney, Mr. Kyle Waite, Dr. Mandi Lopez, Dr. Kevin Kleinow, Dr. Steven Barker, and Dr. Anderson DaCunha.

I want to extend my thankfulness to Mrs. Cheryl Crowder, Mrs. Julie Millard, and Mrs. Thaya Guerry for their precious work and help with my sample analysis and Mr. Michael Broussard for being so kind every time I needed his services.

I would like to thank Mrs. Li-Ju Huang for helping me since the very beginning of my project, and also for sharing with me her wisdom and knowledge.

I would like to acknowledge and extend my gratitude to Dr. Frank Andrews and the Equine Health Studies Program (EHSP) for the financial support, and the EHSP staff Mr. Michael Keowen, Mr. Frank Garza, Ms. Catherine Koch, and EHSP student workers Ms. Elise

Madera, Ms. Katee Carlisle, Ms. Makensie Moore, Mr. Matthew Mcgeachy, Mr. Nicolas Lavie, and Mr. Ross Freeland for helping me in many ways during my studies.

I want to thank Mrs. Jackie Murray and Mrs. Patsy A. Johnson for their help and specially, I would like to Mrs. Jackie Bourgeois for being always so kind and supportive for all these years.

In memory of Dr. Abolghasem Baghian, I thank him for his assistance.

I would like to extend my gratitude to the Associate Dean for Research and Advanced Studies, Dr. Thomas R. Klei and the Associate Dean for Institutional Advancement and Strategic Initiatives, Dr. David F. Senior for the financial support for part of my program.

I would like to express my gratitude to Dr. Alex Baudena and Ms. Sona Chowdhury for their friendship, help, and support. I am deeply grateful to Mr. Andrew D. Augustine, Dr. Jose Mauricio, and Mrs. Blandina Brandao, who were more than my friends and definitely became part of my family.

## TABLE OF CONTENTS

DEDICATION .....	ii
ACKNOWLEDGEMENTS .....	iii
LIST OF TABLES .....	viii
LIST OF FIGURES .....	ix
ABSTRACT .....	x
 CHAPTER 1. A REVIEW OF OPIOID ANALGESIA AND THE MULTIDRUG RESISTANCE (MDR1) GENE AND TRANSPORTER PROTEIN – P-GLYCOPROTEIN (P-GP) .....	1
1.1 Introduction to Equine Pain Management .....	2
1.2 Physiology of Pain and Opioid Analgesia .....	4
1.3 Opioid Drugs .....	5
1.3.1 Definition and Classification .....	5
1.3.2 Mechanism of Action: Analgesia and Side Effects .....	6
1.3.3 Routes of Administration: Advantages and Limitations .....	7
1.4 Methadone Hydrochloride .....	9
1.4.1 History .....	9
1.4.2 Characteristics and Mechanisms of Action .....	9
1.4.3 Current Clinical Use .....	11
1.4.4 Oral Pharmacokinetics .....	12
1.4.5 Intestinal Absorption and Oral Bioavailability .....	14
1.5 Multidrug Resistance (MDR1) Gene and P-glycoprotein (P-gp) .....	15
1.5.1 Transporter Proteins: The ABC Family .....	16
1.5.2 Structure, Expression, and Function of P-glycoprotein .....	17
1.5.3 P-glycoprotein-Mediated Drug Transport .....	20
1.5.4 Regulation of Expression and Activity .....	22
1.5.5 Substrates, Inhibitors, and Enhancers of P-glycoprotein .....	24
1.5.6 Relevance and Modulation of P-glycoprotein .....	26
1.6 <i>In vitro</i> Techniques to Study Protein-Mediated Drug Transport .....	28
1.6.1 Cell Culture .....	28
1.6.2. Ussing Chamber .....	30
1.7 Hypothesis and Objectives .....	31
1.8 References .....	31
 CHAPTER 2. PHARMACOKINETICS OF THE INJECTABLE FORMULATION OF METHADONE HYDROCHLORIDE ADMINISTERED ORALLY IN HORSES .....	41
2.1 Introduction .....	42
2.2 Materials and Methods .....	43
2.2.1 Animals .....	43
2.2.2 Study Design .....	44
2.2.3 Serum Sampling and Clinical Evaluation .....	44

2.2.4 Serum Analysis.....	44
2.2.5 Chemicals .....	45
2.2.6 Sample Preparation.....	45
2.2.7 Instrumentation.....	46
2.2.8 Pharmacokinetics Analysis.....	46
2.2.9 Statistical Analysis .....	47
2.3 Results .....	48
2.4 Discussion .....	48
2.5 References .....	52
 CHAPTER 3. BIOAVAILABILITY AND PHARMACOKINETICS OF ORAL AND INJECTABLE FORMULATIONS OF METHADONE AFTER INTRAVENOUS, ORAL, AND INTRAGASTRIC ADMINISTRATION IN HORSES .....	55
3.1 Introduction .....	56
3.2 Materials and Methods .....	57
3.2.1 Animals .....	57
3.2.2 Study Design .....	58
3.2.3 Serum Sampling and Clinical Evaluation .....	59
3.2.4 Serum Analysis.....	59
3.2.5 Pharmacokinetics Analysis.....	60
3.2.6 Statistical Analysis .....	61
3.3 Results .....	61
3.4 Discussion.....	62
3.5 References .....	67
 CHAPTER 4. IDENTIFICATION AND SEQUENCING OF EQUINE MULTIDRUG RESISTANCE (MDR1) GENE AND DIFFERENTIAL mRNA AND P-GLYCOPROTEIN (P- gp) EXPRESSION ACROSS TISSUES IN HORSES .....	71
4.1 Introduction .....	72
4.2 Materials and Methods .....	73
4.2.1 PART 1. Identification and Sequencing of the Equine MDR1 Gene.....	73
4.2.1.1 Design of Primers .....	73
4.2.1.2 Template .....	74
4.2.1.2.1 Tissue Collection .....	74
4.2.1.2.2 RNA Isolation .....	74
4.2.1.2.3 cDNA Synthesis.....	75
4.2.1.3 Polymerase Chain Reaction (PCR).....	75
4.2.1.4 Plasmid pcDNA 3.1/MDR1 .....	75
4.2.1.5 DNA Sequencing and Blast Analysis .....	76
4.2.2 PART 2. Expression of MDR1 Gene in Equine Tissues.....	76
4.2.2.1 Design of Primers and Probes.....	76
4.2.2.2 Template .....	76
4.2.2.2.1 Tissue Collection .....	76
4.2.2.2.2 RNA Isolation .....	78
4.2.2.2.3 cDNA Synthesis.....	78
4.2.2.3 Real-Time PCR (TaqMan).....	78

4.2.3 PART 3. Expression of P-glycoprotein .....	79
4.2.3.1 Plasmid pGEX-6P-1/MDR1 .....	80
4.2.3.2 Western Blot .....	80
4.2.4 Statistical Analysis .....	82
4.3 Results .....	82
4.3.1 Identification, Sequencing, and Blast Analysis of Equine MDR1 Gene .....	82
4.3.2 Differential MDR1 mRNA Expression in Equine Tissues .....	83
4.3.3 Expression of Equine P-glycoprotein .....	87
4.4 Discussion .....	91
4.5 References .....	94
 CHAPTER 5. THE EFFECT OF P-GLYCOPROTEIN ON METHADONE HYDROCHLORIDE FLUX IN EQUINE INTESTINAL MUCOSA .....	98
5.1 Introduction .....	99
5.2 Materials and Methods .....	101
5.2.1 Animals .....	101
5.2.2 Drugs .....	101
5.2.3 Tissue Preparation .....	102
5.2.4 Ussing Chamber Study Design .....	102
5.2.5 Assessment of Tissue Viability .....	103
5.2.6 Methadone Sample Analysis .....	104
5.2.7 Flux of Methadone HCl Across Jejunal Mucosa .....	105
5.2.8 Histology .....	105
5.2.9 Immunohistochemistry .....	105
5.2.10 Solution pH and Osmolality .....	106
5.2.11 Statistical Analysis .....	106
5.3 Results .....	107
5.3.1 Flux of Methadone HCl Across the Jejunal Mucosa .....	107
5.3.2 Immunohistochemistry Analysis .....	108
5.4 Discussion .....	108
5.5 References .....	121
 CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS .....	125
 BIBLIOGRAPHY .....	129
 APPENDIX: LETTER OF PERMISSION .....	142
 VITA .....	145



## LIST OF TABLES

Table 1.1 Substrates and Inhibitors (*) of P-glycoprotein and Their Therapeutic Use .....	25
Table 2.1 Pharmacokinetic Parameters of Oral Administration of Methadone.....	50
Table 3.1 Formulations and Routes of Methadone Administration in Horses .....	59
Table 3.2 Pharmacokinetic Parameters of Intravenous, Oral, and Intra gastric Administration of Methadone.....	64
Table 4.1 Design of Forward and Reverse Primers (GenBank AY360144).....	74
Table 4.2 Design of Primers and Probes.....	77
Table 4.3 Design of Forward and Reverse Primers (GenBank AY968084).....	79
Table 4.4 Real-Time PCR Reaction Efficiency (%).....	86
Table 4.5 Fold-Difference of Relative Quantitation of MDR1 mRNA Expression in Equine tissues.....	87
Table 5.1 Horse Information – Ussing Chamber Study .....	101
Table 5.2 Ussing Chamber Drug Solutions .....	104
Table 5.3 Flux of Methadone HCl in Equine Jejunal Mucosa.....	112

## LIST OF FIGURES

Figure 1.1 Chemical Structure of Methadone and Alkaloid Opioid Molecules .....	10
Figure 1.2 A Two Dimensional Representation of the Methadone Molecule .....	10
Figure 1.3 Schematic Structure of the Plasma Membrane P-glycoprotein (P-gp).....	18
Figure 1.4 Proposed Mechanism Models for P-glycoprotein (P-gp) .....	21
Figure 2.1 Serum Concentration vs. Time Curves for Oral Administration of Methadone in Horses .....	49
Figure 3.1 Serum Concentration vs. Time Curves for Intravenous, Oral, and Intragastic Administration of Methadone in Horses.....	63
Figure 4.1 <i>Equus caballus</i> Multidrug Resistance P-glycoprotein (MDR1) mRNA Partial cds ....	84
Figure 4.2 BLAST (Basic Local Alignment Search Tool) Analysis Between Partial and Predicted Sequences of the Equine Multidrug Resistance Gene (MDR1) mRNA .....	85
Figure 4.3 Relative Quantitation of MDR1 mRNA Expression in Equine Tissues.....	88
Figure 4.4 Coomassie Brilliant Blue Staining (SDS-PAGE).....	89
Figure 4.5 Western Blot Analysis (P-gp-GST fusion protein) .....	89
Figure 4.6 Western Blot Analysis (Expression of P-gp in Equine Tissues) .....	90
Figure 5.1 Short-Circuit Current (I <sub>sc</sub> ) and Spontaneous Potential Difference (PD) in the Jejunum of Horses .....	109
Figure 5.2 Electrical Conductance (G) and Tissue Resistance (R) in the Jejunum of Horses.....	110
Figure 5.3 Concentration of Methadone in the Mucosal Side of the Ussing Chambers.....	111
Figure 5.4 Flux of Methadone in Jejunal Mucosa of Horses .....	113
Figure 5.5 Immunohistochemical Staining of P-glycoprotein in the Jejunum of Horses .....	114
Figure 5.6 Inter-individual Variability of Immunohistochemical Staining of P-glycoprotein in the Jejunum of Horses (40X) .....	115
Figure 5.7 Immunohistochemical Staining for P-glycoprotein in the Villi of Jejunum of Horses (20X) .....	116

## ABSTRACT

Methadone is a mu-opioid receptor agonist which is a very effective analgesic used to treat moderate to severe acute and chronic pain in humans. Due to methadone's minimal undesirable side-effects in people, we believed it could be of use in horses as an analgesic agent. As found with the majority of lipophilic drugs, absorption of methadone occurs primarily in the small intestine via transcellular transport and its absorption is regulated by P-glycoprotein. P-glycoprotein is a transmembrane transporter protein encoded by the multidrug resistance gene, which is constitutively expressed in the apical membrane of enterocytes of various species. This protein may impair the therapeutic efficacy of oral opioids including methadone, by decreasing absorption through the small intestinal mucosa and altering drug's pharmacokinetics. The overall hypothesis was that the expression of P-glycoprotein in the equine small intestine affects absorption and bioavailability of methadone after oral administration to horses.

*In Vivo* and *in vitro* studies presented here investigated the oral pharmacokinetics of methadone, expression of the multidrug resistance (MDR1) gene, and the role of intestinal P-glycoprotein on methadone flux or transport in equine jejunal mucosa. The contribution of this protein to *in vivo* absorption of this opioid drug after oral administration in horses is evaluated.

Oral administration of methadone hydrochloride to healthy horses showed rapid absorption, reaching high serum concentrations without typical undesirable opioid-induced side effects. Drug absorption appears to be limited in the small intestine, supported by the observed low drug serum concentrations, low area under the drug serum concentration vs. time curve, and low drug bioavailability after intragastric administration. In addition, methadone was absorbed by oral mucosa and may be an important way that methadone gains entrance into equine plasma.

Multidrug resistance (MDR1) gene expression was determined in several different tissues including those of the small intestine of horses. High MDR1 mRNA levels mainly in the duodenum and jejunum of horses may explain, at least in part, the limited intestinal absorption of methadone *in vivo*. P-glycoprotein, located in the apical membrane of epithelial intestinal cells of jejunum in horses impairs the flux of methadone across the intestinal mucosa and its drug efflux activity is minimized by verapamil HCl, a P-glycoprotein inhibitor. Therefore, these studies confirmed that the expression of P-glycoprotein in the equine small intestine affects absorption and bioavailability of methadone after oral administration to horses.

**CHAPTER 1.**  
**A REVIEW OF OPIOID ANALGESIA AND THE MULTIDRUG RESISTANCE (MDR1)**  
**GENE AND TRANSPORTER PROTEIN – P-GLYCOPROTEIN (P-gp)**

## 1.1 Introduction to Equine Pain Management

Pain is defined by the International Association for the Study of Pain since 1979, as “an unpleasant sensory and emotional experience or perception associated with actual or potential tissue damage”<sup>1,2</sup>. In horses, pain is one of the major factors responsible for decrease or loss of animal performance and significant amount of money spent in treatments, remaining a substantial issue with huge socio-economical impact on the equine industry<sup>3</sup>. Although proper analgesia is required to minimize suffering and improve recovery time for all species, pain management is still unsatisfactory in horses due to the difficulties in assessing pain in animals and to the majority of available analgesic is associated with relevant negative side effects<sup>4</sup>.

Non-steroidal anti-inflammatory drugs (NSAID) reduce inflammation and pain by suppressing the cyclooxygenase (COX) mediated production of prostaglandin E (PGE<sub>2</sub>). Compared to traditional NSAID such as phenylbutazone and flunixin meglumine, COX-2 selective inhibitors are associated with reduced gastrointestinal and other side effects and have minimal effect on platelet activity. However, most of the NSAID drugs currently available for use in horses are still nonselective COX inhibitors<sup>5,6</sup>.

Lidocaine is a local anesthetic with some analgesic properties. Administered as a bolus followed by a constant rate infusion due to its short half-life, lidocaine provides systemic analgesia, scavenges free radicals, and increases gastrointestinal motility. Spinal and supraspinal mechanisms are suggested to be involved, but the exact mechanism of action of this drug is not fully understood. Usually in association with other drugs, in particular opioids, this drug can potentially relieve neuropathic pain in humans<sup>5,6</sup>.

Alpha-2 agonists are potent analgesics to treat acute pain and are potential inhibitors of central hyperalgesia when systemically administered. However, these agents are not usually the

first choice for analgesia due to their side effects on the cardiopulmonary (pronounced decreases in heart rate and cardiac output, and respiratory depression), gastrointestinal (decrease of motility), and metabolic system remain of concern<sup>5,6</sup>.

Ketamine is a dissociative anesthetic with antihyperalgesia properties due to its action as an antagonist at the N-methyl-D-aspartate (NMDA) receptor. Ketamine is most effective when administered in association with other analgesic drugs, potentiating the antinociceptive effects of opioids and alpha-2 agonists<sup>5,6</sup>.

Gabapentin (alpha-2- $\delta$  ligand) is a gamma-aminobutyric acid (GABA) analog that prevents the release of nociceptive transmitters by binding to the alpha-2- $\delta$  subunit of voltage-gated calcium channels. Clinically used as anticonvulsivant, this drug is also an effective analgesic in patients with neuropathic or chronic pain syndromes. However in horses, gabapentin was demonstrated to be poorly absorbed after oral administration<sup>5,6</sup>.

Opioids are potential analgesic agents which decrease perception of pain, decrease reaction to pain, and increase pain tolerance. Frequently used in multimodal analgesia, some authors suggest opioids are by far, the most appropriate medication particularly for the treatment of moderate to severe acute or chronic pain<sup>5,6</sup>. The use of opioids is limited in horses since they have adverse side effects including excitement, increased locomotor activity, and decrease gastrointestinal motility after intravenous administration<sup>4,6</sup>. Butorphanol, a synthetic opioid, is probably the most widely used in horses for pain relief and sedation. This synthetic agonist-antagonist opioid provides mild analgesia and less excitement compared to pure opioid agonists, but it is a very short acting analgesic drug (short half-life)<sup>1,5,6</sup>. Alternative methods for pain management include new or current pharmacological agents with alternative routes for drug

administration are being investigated in horses to achieve satisfactory therapeutic effects with minimal opioid-induced side effects<sup>7-11</sup>.

## **1.2 Physiology of Pain and Opioid Analgesia**

The mechanism of pain involves a complex sequence of biochemical and electrical events within the brain which begins with tissue damage and release of endogenous transmitters (substance P, glutamate, and others) followed by transduction, transmission, perception and modulation of the pain stimulus. The nociception process starts in the periphery with the stimulation of high-threshold specialized sensory nerve endings or receptors (nociceptors), which respond to potential or damaging (noxious) stimuli including mechanical, thermal or chemical changes above a set threshold. The energy of the stimulus is converted into an electrical impulse (transduction) and transmitted from the periphery to the dorsal horn of the spinal cord through fast-conducting myelinated A-delta and slow-conducting unmyelinated C primary afferent fibers (transmission). A-delta fibers are responsible for fast nerve impulse conduction of sharp and localized pain (first pain) while C fibers are related to slow stimulus conduction of a more burning type and poorly-localized pain (second pain). These fibers supply skin and subcutaneous tissues, periosteum, joints, muscles, and viscera. Nociceptive and also non-nociceptive information may be conveyed from the spinal cord to different parts of the brain by multiple pathways including lateral and medial spinothalamic tracts, lateral spinocervical and medial spinoreticular tracts, lateral dorsal column-postsynaptic tract, spinomesencephalic tract, and the propriospinal system. Along with temperature fibers, pain stimuli cross the spinal cord and ascend to the brain preferably via the lateral spinothalamic tract. Ascending fibers in this tract terminate primarily in the brain stem and thalamus and relay the impulse or information to the



cerebral cortex for pain localization (perception). The reticular and limbic systems are also involved in the process of pain perception<sup>1,12-14</sup>.

Modulation of the pain stimulus occurs as an innate response of the body to relieve pain sensation through endogenous descending analgesic systems (noradrenergic, serotonergic, and endogenous opioid systems). It is originated in the brain stem and modifies impulses or inhibits nociceptive transmission at the spinal cord level by neuromodulator release (norepinephrine/noradrenaline [NA], serotonin [5-hydroxytryptamine or 5-HT], and endogenous opioids)<sup>1,6,12-15</sup>. Activation of these pain inhibitory descending systems markedly modifies the release of glutamate, GABA, and glycine, altering the transduction and or transmission and therefore, reducing pain perception<sup>15</sup>.

Endogenous opioid peptides ( $\beta$ -endorphin, dynorphin, enkephalin, and endomorphins) function by specific receptor binding at the spinal cord (dorsal horn), brainstem, medulla, periaqueductal grey substance, thalamus, limbic system, and cerebral cortex. In addition to the intrinsic analgesic mechanism, exogenous opioids are one of the most potent and extensive analgesic drugs class used to treat moderate to severe, persistent or chronic, and neuropathic pain<sup>6,12-14,16-19</sup>.

### 1.3 Opioid Drugs

#### 1.3.1 Definition and Classification

Opioids are all opium (*Papaver somniferum*) alkaloid derivatives –natural, synthetic or semisynthetic, and agents which effects are blocked by classical opioid antagonists. They usually have in their chemical structure a phenol, aromatic ring, and ionized amine groups<sup>1</sup>.

Based on their receptor affinity and intrinsic efficacy, opioid drugs are classified as agonists and/or antagonists. Among agonists, opioids are separated as strong or full (pure)

agonist showing high receptor affinity and maximum intrinsic activity (morphine, hydromorphone, oxymorphone, heroin, methadone, levomethadyl acetate, meperidine, and fentanyl), partial agonists with some affinity and mild or moderate activity (buprenorphine, codeine, oxycodone, phenylheptylamines, loperamide), and mixed agonist-antagonists binding more than one receptor (butorphanol, pentazocine, and tramadol). Opioid antagonists are molecules that have affinity for opioid receptor but no intrinsic activity; they block or reverse pharmacologic effects of opioid agonists by competing for the same receptor sites. As a pure antagonist, naloxone is the best example<sup>20</sup>.

### 1.3.2 Mechanism of Action: Analgesia and Side Effects

Analgesia is induced by opioid binding to specific receptors mainly located in the spinal cord (dorsal horn) and brain, but also found in synovial fluid and the gastrointestinal tract<sup>1,21</sup>. Opioid receptors are pharmacologically classified as mu ( $\mu$ ), delta ( $\delta$ ), or kappa ( $\kappa$ ), but they can be also named OP<sub>3</sub>, OP<sub>1</sub>, and OP<sub>2</sub>, respectively, according to the International Union of Pharmacology (IUPHAR). The IUPHAR nomenclature indicates the nature of the endogenous ligand (OP – opioids) and chronological order of discovery<sup>1,22</sup>. Opioids exert their action via effector proteins by binding to G-protein coupled opioid receptors. They reduce neurotransmitter release from primary afferents, especially substance P, by closing voltage-gated Ca<sup>+2</sup> channels on presynaptic neuronal terminals mainly in the dorsal horn or by increasing K<sup>+</sup> channel conductance and inhibiting postsynaptic neurons (hyperpolarization). At the cellular level, opioids inhibit adenylyl cyclase thereby decreasing production of cyclic AMP (cAMP) consequently inhibiting nociceptive or excitatory neurotransmitter release. The lack of neurotransmitters makes the neurons less likely to relay stimulus transmission, inhibiting the process of pain. Centrally, opioids activate the descending endogenous antinociceptive system

that modulates nociception in the dorsal horn via endogenous opioids, serotonin and norepinephrine release<sup>1</sup>.

In addition to potent analgesic effects, opioids induce relevant side effects on the central nervous system and peripheral organs. Most of the opioids act at the mu receptor which is responsible for the analgesic properties and the major side effects like euphoria, sedation, miosis, tolerance, and respiratory depression. Delta receptors are associated with some analgesia (spinal) and euphoria while (kappa) receptors produce miosis, sedation and dysphoria in addition to analgesia (spinal)<sup>1,13,16,17,23-26</sup>. Constipation, ileus, and occasionally abdominal pain are adverse side effects due to their action on the gastrointestinal tract<sup>21</sup>.

Tolerance (gradual loss of effects) and physiological withdrawal symptoms (physical dependence) are potential side effects due to repeated administration of opioids. Opioids suppress immune functions after acute (single) or chronic (repeated injections over time or pellet implantation) administration involving mainly central opioid receptors. Pre- or co-administration of opioid antagonist is suggested to attenuate or reverse opioid-induced immune suppression<sup>1,27</sup>.

Presence or absence of pain has a major influence on the response and side effects induced by opioid drugs. Usually opioid-induced side effects are attenuated in horses and cats under pain stimulation<sup>13</sup>.

### **1.3.3 Routes of Administration: Advantages and Limitations**

Opioids have been used in horses for seventy years. They are usually administered in combination with tranquilizers and sedatives to minimize sympathetic stimulation and central nervous system (CNS) excitation<sup>15-17,28-31</sup>.

Excitement, decreased intestinal motility, and increased locomotor activity are the most common side effects associated with systemic administration of opioids in horses<sup>28-31</sup>.

Excitement may also occur in cats, ruminants, and swine, and is usually associated with mydriasis, tachycardia, hypertension, and sweating<sup>1,13,17,24-26</sup>. Muscle rigidity and central nervous system depression associated with miosis, bradycardia, and hypothermia are more commonly recognized in primates, dogs, and rabbits<sup>1,13,19</sup>. Opioid-induced gastrointestinal effects depend on the specific drug agent and vary among individuals. In horses, gastrointestinal side effects are considered the most important limitations for opioid usage. Opioid drugs decrease the propulsive activity of the gastrointestinal tract by inhibiting peristalsis, increase transit time leading to constipation, while increasing tone in intestinal smooth muscle<sup>21</sup>.

Alternative routes to systemic opioid administration seek to increase therapeutic efficacy and minimize adverse side effects. Subarachnoid or epidural injection of opioids including morphine, hydromorphone, or methadone produces long lasting and potent analgesia without CNS excitement in horses. Nevertheless, the segmental analgesic effect of these routes is limited to the hind limbs and thoracic wall<sup>1,7-12,14,32</sup>. Transdermal and subcutaneous routes have not been extensively studied in horses. However, pharmacokinetics (PK) data of fentanyl patches suggested that therapeutic antinociceptive effects or analgesic drug plasma concentrations are not achieved in horses<sup>33</sup>.

Oral administration is an easy, low cost, non-invasive, and effective alternative route for several drugs including opioids. It usually provides potent analgesia with minimum or less intense side effects in the majority of human patients. Methadone is one of the main opioids clinically used by the oral route to treat acute and/or chronic pain in cancer patients<sup>34-40</sup>. In horses, oral methadone could circumvent the most important drug induced side effects and represent a potential advancement for equine pain management. However, the oral administration of methadone has not been thoroughly evaluated in horses.

## 1.4 Methadone Hydrochloride

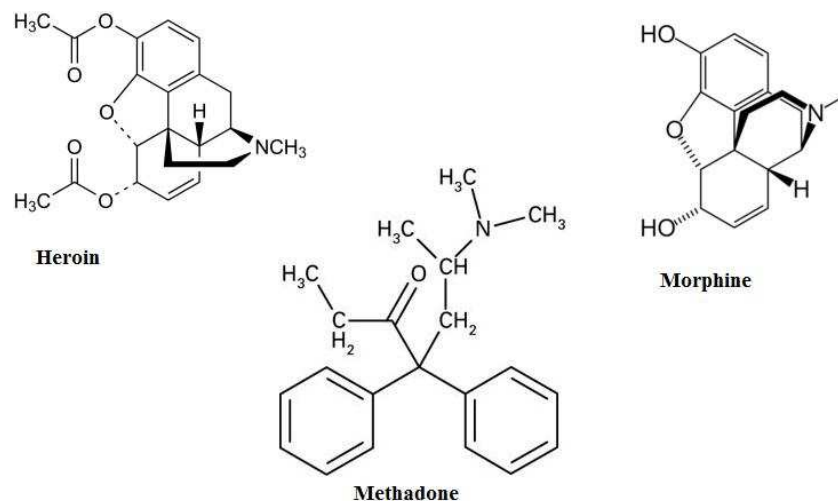
### 1.4.1 History

Methadone is currently considered as a schedule II narcotic agent by the U.S. Drug Enforcement Administration (DEA). First synthesized in Germany in 1937, methadone was developed during World War II as an alternative to morphine mainly due to the scarcity of this drug. Methadone was used because of its ease administration and less potent addictive effects. Although dissimilar in structure from morphine, heroin or other opioid alkaloids, methadone also acts on opioid receptors and produces similar effects (Figure 1.1). The synthetic substance of methadone was initially called Hoechst 10820 or polamidon and it was introduced into the United States as an analgesic under the trade name Dolophine in 1947. This name originated from the word *Dolphium* which means in Latin *dol* = *dolor* = "pain" and *phium* = *phine* = "end". Methadone was mainly used to treat drug addiction in people in the first two decades, and it was not until 2001 that regulations expanded the use of this drug as an analgesic agent. At present, methadone is one of the major opioid drugs recommended for management of malignant and nonmalignant chronic pain. Methadone has a long duration of action, less expensive, and less side effects than other opioids. However, since methadone still carries the stigma of being related to drug abuse and addiction, a strong resistance still exists towards its use<sup>41,42</sup>.

### 1.4.2 Characteristics and Mechanisms of Action

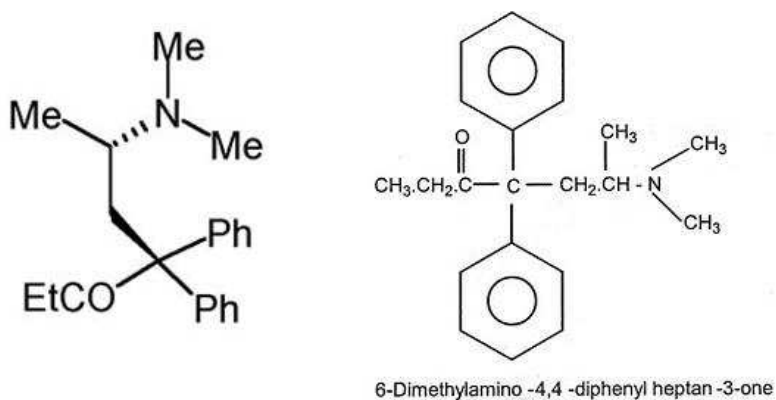
Methadone (6-dimethylamino-4,4-diphenyl-heptan-3-HCl) is an alkaloid compound with a naturally occurring nitrogenous organic molecule (amine) at the six carbon which is responsible for its pharmacological effects. As a diphenylpropylamine derivate narcotic, this drug possesses the simplest chemical structure among opioid drugs. Methadone is a weak base (pka=9.2), highly lipophilic, and water soluble with molecular weight of 309.445 g/mol. When

combined with hydrochloride acid (HCl), it has molecular weight of 345.91 g/mol and pka of 8.25 (Figure 1.2)<sup>38,42,43</sup>.



**Figure 1.1 – Chemical Structure of Methadone and Alkaloid Opioid Molecules**

Dissimilar structure between opioid alkaloid drugs methadone, morphine, and heroin.



**Figure 1.2 – A Two Dimensional Representation of the Methadone Molecule**

A two dimensional illustration of the methadone molecule. It bends into a structure similar to morphine and the piperidine ring to fit into the opiate receptors. Chemical structure of methadone:  $C_{21}H_{27}NO \cdot HCl$  (6-dimethylamino-4,4-diphenyl-heptan-3-one [HCl]). Data is from Payte et al., 2001<sup>42</sup>.

Methadone is a potent synthetic mu-opioid receptor agonist approximately equipotent to morphine in analgesic effects. It produces less sedation in dogs and more ataxia in horses. Methadone comprises particular characteristics (non-opioid actions) that enhance its analgesic property and makes it different from other opioid drugs. Also named OP<sub>3</sub> agonist, this drug is commonly used as a racemic (*R/S* or *d/l*) mixture<sup>38,44</sup>. Like all other opioids, the *R*-enantiomer or *l*-isomer of methadone binds more specifically to cell surface OP<sub>3</sub> or mu receptors eliciting most of the therapeutic and unwanted side effects. The opioid receptor is a G-protein coupled (metabotropic) receptor which conveys the signal through effector proteins such as adenylyl cyclase and phospholipases. The *R/l* isomer is approximately 8 to 50 times more potent than the *S/d* isomer. The *S*-enantiomer or *d*-isomer binds to N-methyl-D-aspartate (NMDA) ligand-gated ion channel (ionotropic) glutamate receptors which convey the signal by altering cell membrane potential or ionic composition<sup>45-47</sup>. As an NMDA receptor antagonist, methadone is also able to reduce the nociceptive response. This antagonism blocks opioid tolerance and depolarization of spinal cord neurons preventing hyperalgesia, central sensitization or “wind-up” phenomenon. Furthermore, methadone functions as a serotonin- and norepinephrine-reuptake inhibitor, contributing to the antinociceptive pathway. Methadone inhibits the monoamine oxidase enzyme (MOA) which is responsible for the uptake and monoamines degradation, and allows the catecholamines to remain longer in the circulation<sup>20,36,39,42</sup>.

#### 1.4.3 Current Clinical Use

In humans, methadone is in the frontline for moderate to severe pain management showing great clinical relevance in the treatment of pain due to cancer and suppression of opioid-agonist abstinence syndrome in narcotic-dependent patients. Used in multimodal pain

management approaches or by oral administration, methadone does not induce either severe side effects or opioid dependence (addiction) as described for other opioid drugs<sup>18,34,37,40</sup>.

In veterinary medicine, methadone is used as an analgesic agent in combination with other drugs due to its narrow margin between analgesia and stimulation or excitation. Although alternative routes have been investigated to enhance analgesic effects and minimize drug-induced side effects, little is known about oral methadone in horses<sup>32,48</sup>.

#### 1.4.4 Oral Pharmacokinetics

Pharmacokinetics of methadone has been widely studied in humans, but less extensive in other species such as felines and canines<sup>43,44,46,49-52</sup>. Despite large individual variability in therapeutic response, drug toxicity, and drug PK in people, oral methadone has excellent absorption and high oral bioavailability (70-100%), long duration of action with long elimination half-life ( $T_{1/2}$  or HL) of 20 to 24h, and low risks of induced side effects<sup>37,38,40,46,47,49</sup>. Bioavailability (F) represents the percentage of drug absorbed and available to produce systemic effects (percentage of drug that reaches the systemic circulation) based on the area under the drug plasma concentration-time curve (AUC). The AUC represents the amount of drug in the blood during drug disposition and it is an indirect indicator used in the calculation of bioavailability<sup>1,53</sup>.

In horses, information regarding the pharmacokinetics of methadone is limited. Recent investigations of a single oral administration of methadone in horses revealed good drug absorption, high serum drug concentrations, and no induced side effects<sup>54</sup>. The therapeutic or analgesic concentrations of methadone has not been established in horses; however, drug serum concentrations after a single oral dose up to 0.4 mg/kg were higher than the therapeutic blood concentrations of methadone for acute pain in people which were reported to be between 33 and



59ng/ml<sup>54-56</sup>. Methadone showed large volume of distribution ( $V_d$ ) in horses, which represents the apparent volume of the plasma compartment where the drug is distributed. It is an indicator of drug distribution and affinity for tissue, and a possible indicator of drug protein binding. Methadone binds primarily to  $\alpha$ 1-acid glycoprotein, but also to albumin and lipoproteins, and it may accumulate in tissues with continued dosing<sup>1,53,54</sup>.

Drug elimination can occur either by metabolism or excretion. Methadone undergoes oxidative N-demethylation in the smooth endoplasmic reticulum – microsome (enterocytes and hepatocytes) as its major pathway of hepatic biotransformation producing two inactive metabolites as analgesics, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidene (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP). Biotransformation occurs predominantly by the cytochrome P450 (CYP) enzymes, mainly CYP3A4 isoform and to a lesser extent CYP2D6 in humans and rats<sup>16,45</sup>. CYP3A4 is an inducible enzyme that undergoes auto-induction by chronic administration of methadone resulting in greater hepatic metabolism and first-pass effect<sup>47</sup>. Several isoforms including CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and particularly CYP2B6 have been suggested to take part in drug metabolism<sup>42,43,46,57-59</sup>. A minor pathway, ketone reduction, was reported to contribute to less than 25% of methadone metabolism and it was associated with the production of active metabolite methadol, which is then N-demethylated to normethadol which retains some analgesic activity<sup>60</sup>. The metabolism of methadone is not fully characterized and little is known about these enzymes in domestic animals. Unlike in humans, CYP3A4 may not be the major metabolizing enzyme and EDDP and EDMF are considered minor metabolites in dogs<sup>52</sup>.

Methadone has a long elimination half-life of 15 to 40 hours in humans and intact methadone and metabolites are mainly excreted from the body by the liver, intestine (bile and

feces), and kidneys with urine accounting for 20 to 50% of the total elimination<sup>38,47,52</sup>. Drug elimination is estimated by drug clearance (Cl) that measures the hypothetical volume of blood cleared per minute to eliminate the total concentration of drug without distinction or differentiation of the elimination process. Elimination half-life is more an indicator of drug persistence in the body, but it can be used as an indirect indicator of drug elimination. Half-life represents the time required for the plasma drug concentration decrease by 50%. Drug elimination can be directly affected by half-life, patient sex, urine pH, and duration of treatment or autoinduction of the hepatic enzyme system<sup>42,53</sup>.

#### **1.4.5 Intestinal Absorption and Oral Bioavailability**

Intestinal drug absorption is an important determinant for oral bioavailability. Drug transport across the small intestine epithelium can occur via a passive transcellular or paracellular process mainly in the jejunum due to presence of villi and microvilli which increase the absorptive surface area of the apical membrane. In general, more lipophilic drugs such as methadone preferably diffuse through the transcellular route<sup>42,51,61,62</sup>.

Drug absorption is primarily dependent on physicochemical properties of the drug such as chirality, lipophilicity and solubility, acid-dissociation/ionization (pka), stability, partition coefficient (log P), crystal form, hydrogen bonding capacity, molecular size and weight. However, intestinal absorption is a complex process also affected by factors including physiological and pathophysiological characteristics (intestinal motility, permeability, intestinal pH, and metabolic enzyme activity) and genetic factors<sup>42,62</sup>.

Methadone fulfills specific criteria or requirements related to its physicochemical characteristics which determine a successful drug molecule consistent with high absorption and bioavailability ('Rule of Five'). Methadone fits the 'Rule of Five' meeting four criteria; no

hydrogen-bond donors, single oxygen and nitrogen molecules, octanol/water partition coefficient (log P) less than 5, and low molecular weight below 500 Da, which are all associated with high drug solubility and permeability<sup>63</sup>.

Among genetic factors, transporter proteins are recognized as important “regulators” of drug transport across the cell membrane with critical impact on oral bioavailability<sup>42,62</sup>. The multidrug resistance gene plays a significant role in the absorption and disposition of many drugs including methadone<sup>64</sup>. However, little is known about transporter proteins in species other than humans<sup>51</sup>. In horses, expression of the multidrug resistance gene and its interference on oral opioid pharmacokinetics, mainly absorption or disposition of methadone, has not been yet investigated.

### **1.5 Multidrug Resistance (MDR1) Gene and P-glycoprotein (P-gp)**

Due to the recognition of the huge impact of drug efflux transporters on the pharmacological behavior of many clinically used drugs, pharmacogenomics studies have enhanced the understanding and approach regarding human and veterinary therapeutics.

Transporter proteins are suggested to limit several pharmacological therapies due to their interference with the pharmacokinetics and pharmacodynamics of a wide range of drugs. Able to export compounds or xenobiotics out of cells, these transporters are in general, associated with decreased absorption and low plasma drug concentrations by limiting drug entry into the body. These proteins critically affect drug disposition also by increasing metabolism and excretion of drugs. In addition, transporter proteins are associated directly or indirectly with diverse processes and diseases especially in humans<sup>65-68</sup>.

### 1.5.1 Transporter Proteins: The ABC Family

The adenosine triphosphate (ATP)-Binding Cassette (ABC) superfamily of membrane transport proteins is one of the largest classes across all species and comprises a large number of functional transmembrane proteins including the multidrug resistance (MDR) gene, multidrug resistance associated protein (MRP), and breast cancer resistant protein (BCRP). These proteins are considered energy ATP-dependent efflux transporters by actively exporting compounds out of the cells promoting a unidirectional (basolateral to apical) flux<sup>69,70</sup>.

The MDR gene 1 (MDR1), also known as ABCB1, belongs to the subfamily B, member 1 of the ABC superfamily and it is one of the best characterized transporter in humans. The MDR name was conferred after the phenomenon of tumor cells to resist to multiple chemotherapeutic drugs was observed when P-glycoprotein was overexpressed<sup>65-67,69,71-73</sup>.

The MDR1 gene codes for the P-glycoprotein (P-gp) which has been extensively explored in humans. Although the veterinary medicine is in the early stages of understanding the role of P-gp in drug response, this is probably the most widely recognized protein with clinical impact in animals<sup>69,70</sup>.

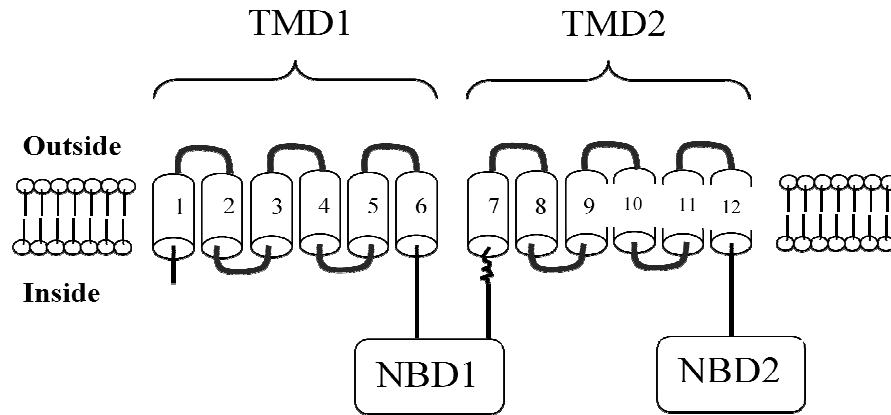
MDR genes comprise two isoforms in humans and dogs (MDR1 and MDR2 or MDR3) and three isoforms in rodents (mdr1 or mdr1b, mdr2, and mdr3 or mdr1a) with considerable identity and homology among them. Human MDR1 and MDR2 genes share 76% of identical coding sequence, but only the MDR1 is responsible for multidrug resistance. Between species, rodent mdr1a and mdr1b serve together a similar function as the single human MDR1 gene and show 88% identity with the human gene. The mdr2 gene in rodents is more homologous to the human MDR2, the isoform located almost exclusively in the liver<sup>65,71,74</sup>. MDR genes are located

on human chromosome 7, mouse chromosome 5, and Chinese hamster chromosome 1, and canine chromosome 14<sup>65,69,71,72,74</sup>.

### 1.5.2 Structure, Expression, and Function of P-glycoprotein

P-glycoprotein (P-gp) is a large (130-180 kDa) protein first described in 1976 by Juliano & Ling in Chinese hamster ovary cells selected in culture for colchicine resistance<sup>75</sup>. Highly phosphorylated and glycosylated, this plasma membrane or integral efflux protein encoded by the MDR gene (MDR1 in humans) can modulate membrane permeability of a number of apparently unrelated drugs<sup>75</sup>.

In eukaryotes, P-gp consists of a dimer structure with 1280 amino acids, 12 hydrophobic transmembrane alpha helices domains (TMD), and two ATP-binding domains with 65% of amino acid similarity (Figure 1.3). The two homologous dimers of this protein are connected by the “linker region”, a central sequence that comprises phosphorylated serine residues and the first extracellular loop is heavily N-glycosylated. The TMDs are the sites where drug molecules or specific compounds cross the membrane and contain two intracellular NH<sub>2</sub>- and COOH-termini. Also called nucleotide-binding domain (NBD), the ATP-binding domains are relatively hydrophilic intracytoplasmic loops encoding ATP sites which are essential for proper functioning of this protein. Each NBD is composed of three conserved consensus sequences or regions, Walker A motif, Walker B motif, and C region or “S signature” which are directly involved in the binding and hydrolysis of nucleotides<sup>65,70,74,76,77</sup>. Motif A can also be recognized as “glycine-rich loop” or “P-loop” and it is involved in binding ATP phosphates while Motif B usually interacts with nucleotide phosphates. Phosphorylation on different sites of this protein, through several kinases including kinase C and cAMP-dependent protein kinase A, is believed to modulate protein activity and therefore, the level of drug resistance<sup>71</sup>.



**Figure 1.3 – Schematic Structure of the Plasma Membrane P-glycoprotein (P-gp).**

Structure of the P-glycoprotein molecule characterized by two transmembrane domains (TMD1 and TMD2) containing six transmembrane segments and a hydrophilic ATP- or nucleotide-binding domain (NBD1 and NBD2) each. Data is from Clarke, 2006<sup>78</sup>.

Expression, location, and function of P-glycoprotein vary among individuals and between species<sup>69</sup>. Usually overexpressed in tumor cells, this protein is strongly correlated with treatment failure and poor prognosis in several types of cancer<sup>71,75</sup>. In humans, high levels of P-gp are detected in a broad range of tumors derived from normal tissues that regularly express this protein<sup>73</sup>.

P-glycoprotein is also constitutively expressed predominantly in the cells lining the luminal space of normal tissues and organs including kidney, liver, intestine, and pancreas. Due to wide distribution and high expression levels mainly in organs with specialized excretory, secretory, and barrier functions, P-gp plays a role in the detoxification and protective mechanism against xenobiotics, metabolites, and potentially toxic compounds<sup>68,79-84</sup>. As a result of its anatomical location, P-gp may function in three main ways, limiting drug entry, enhancing drug metabolism, and/or increasing drug elimination. Expressed in the luminal (apical) membrane of epithelial intestinal cells (enterocytes), P-gp limits or prevents drug entry into the systemic blood circulation after oral administration by transporting or exporting compounds out of the intestinal epithelial cells and back into the intestinal lumen. For drugs that actually reach the blood circulation, P-gp contributes to their metabolism and/or elimination into bile and urine as a result of protein expression in the canalicular membrane of hepatocytes and apical surface of biliary ductules, cells in the renal medulla, renal cortex and apical membrane/brush border of proximal tubule and collecting ducts cells in the kidney. Expressed in capillary endothelial cells at blood-tissue barriers, P-gp limits entry and accumulation of potential harmful circulating compounds in tissues within brain and central nervous system (CNS), fetus, and the testis<sup>67,68,71,76,85</sup>. In the secretory glands of the pregnant endometrium (placenta) and adrenal cortex, P-gp is associated with physiological steroid secretion as well as protection of the fetus against intoxication.

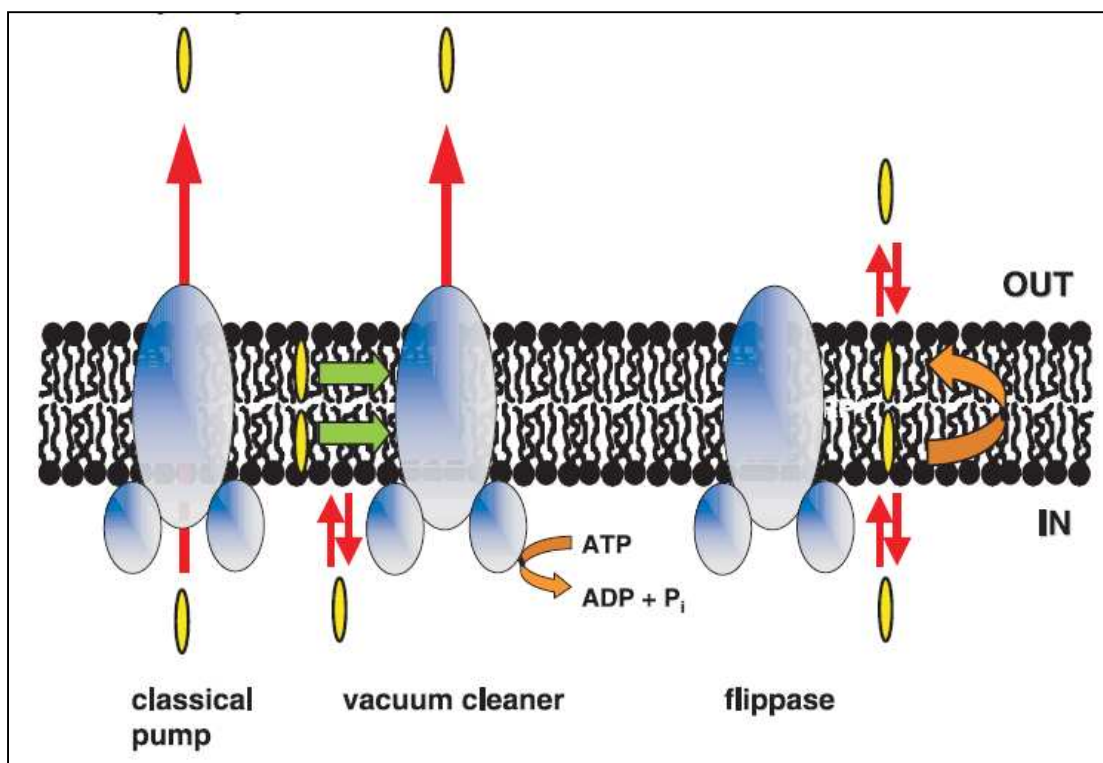
Among white blood cells or leukocytes, natural killer cells and CD4+ / CD8+ lymphocytes have the highest expression of P-gp, suggesting its involvement in cell-mediated cytotoxicity<sup>67,68,71,76</sup>. Although the functions of P-gp are not yet completely understood, this protein is suggested to be involved with further physiological roles including cell signaling, regulation of membrane processes, and membrane composition<sup>66,68</sup>.

The clinical interest in transporter proteins such as P-gp has progressively increased in both human and veterinary medicine. P-gp seems to be involved in the mechanisms underlying drug resistance not only in chemotherapy, but also in therapies utilizing other drugs<sup>86</sup>. Likewise, an improper function of MDR1 and P-gp may also have a negative clinical impact. Although not associated with phenotypic characteristics, deficiency, polymorphism or mutation of MDR1 is hypothesized to increase plasma drug concentrations, to cause drug intoxication, and to increase the susceptibility of diseases in people including Parkinson's disease, multidrug resistance epilepsy, and inflammatory bowel disease<sup>65,66,68</sup>.

### 1.5.3 P-glycoprotein-Mediated Drug Transport

After passive uptake across the intestinal epithelial cell membrane and protein-compound/drug interaction occurs (entry of substrate into the protein binding pocket), conformational (allosteric) changes in the membrane domain are required to reduce binding affinity and expose the binding site to the extracellular space to allow release of the drug into the intestinal lumen. Subsequent to drug release, the protein returns to its original configuration (drug-binding site to the extracellular side). Three models are currently proposed to explain the P-gp mediated drug transport, namely the “classical pump”, “flippase” and “vacuum cleaner” models (Figure 1.4).





**Figure 1.4 – Proposed Mechanism Models for P-glycoprotein (P-gp).**

Classical pump or classical model, vacuum cleaner, and flippase as the three proposed models of P-gp-mediated drug transport. In the classical pump model, polar substrates are transported from the aqueous phase on one side to the other of the membrane through a hydrophilic path formed by the transmembrane regions of the P-gp. In the vacuum cleaner model, drugs first partition into the lipid bilayer, interact with P-gp, and are effluxed into the aqueous phase on the extracellular side. In the flippase model, drugs partition into the membrane, interact with the P-gp in its drug-binding pocket within the cytoplasmic leaflet, and are flipped to the outer membrane leaflet to partition into the extracellular aqueous phase. Data is from Sharom, 2006<sup>77</sup>.

In the “classical pump” or classical model, P-gp acts as a transmembrane hydrophobic channel or pore between the aqueous phase on one side to the other side of the membrane allowing transport of polar substrates from the cytoplasm directly to the extracellular space. In the “flippase model”, P-gp encounters the substrate in the inner leaflet of the plasma membrane (the leaflet closest to the cytoplasm) and flips it to the outer leaflet to diffuse into the extracellular medium. The “hydrophobic vacuum cleaner” model suggests that the protein binds directly the substrate in the lipid bilayer of the plasma membrane and then the protein pumps the substrate out of cell back into the extracellular space<sup>71,76,77</sup>.

An alternative mechanism proposes that P-gp indirectly reduces intracellular accumulation of certain substrates by acting as a proton pump or a chloride channel and affects the intracellular pH and/or the plasma membrane electric potential of the cell. However, this model is the least discussed and does not agree with some of the most recent studies<sup>71</sup>.

#### **1.5.4 Regulation of Expression and Activity**

Transport activity and expression of P-gp are still not completely understood; however, a number of factors are already recognized to alter protein expression and function including physiological signals and endogenous compounds (hormones), pathological conditions, and external factors<sup>70</sup>. It has been described that ABCB1 is subject to hormonal and immunological regulation and is also dependent on age<sup>67</sup>.

MDR1 and P-gp activity can be regulated at several levels by endogenous compounds, xenobiotics, and naturally occurring substances that work as inducers or inhibitors. This regulation can occur by direct drug-protein binding, ATPase activity inhibition, and/or membrane reorganization. Endogenous compounds associated with the inflammatory cascade are described to affect negatively P-gp activity. Endothelin, nitric oxide, and cytokines -tumor

necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, IL-2, and interferon (IFN)- $\gamma$  and external agents that activate these inflammatory mediators, such as radiocontrast agents, aminoglycosides, and heavy metals are examples that lead to decreased P-gp activity<sup>69,70</sup>. N-glycosylation of P-gp appears to confer molecular stability and its inhibition is suggested to decrease protein activity, as well as P-gp phosphorylation. Lipid bilayer composition, presence of cholesterol, and protein interaction with the lipid membrane are essential for the catalytic function of the NBD, basal ATPase activity and therefore, stimulation of P-gp activity. Additionally, it has been shown that depletion of cholesterol decreases MDR1/P-gp activity<sup>72,77</sup>.

In vivo regulation of P-gp activity seems to have species-specific mechanisms. It may be closely associated with molecular events of carcinogenesis, certain xenobiotic treatments, levels of steroid hormones such as estrogen and progesterone, and other physiopathological situations including cholestasis or carbon tetrachloride intoxication<sup>71</sup>.

Expression of P-gp seems to be tied in with the individual innate immune response and results from up-regulated transcription, epigenetic modification, mRNA stabilization, and translation initiation<sup>69,87</sup>. Oxidative stress, hypoxia, inflammation, oncogene transfection, cell differentiation or proliferation and certain diseases may up- or down-regulate MDR1 and P-gp expression, while environmental factors including xenobiotics, heavy metal salts, heat shock, and UV radiation are suggested to up-regulate protein expression<sup>69,70,72,77</sup>.

The cellular and molecular regulation mechanisms of P-gp expression are still not completely understood; however, its transcriptional regulation involves species-specific ligand-activated (orphan) nuclear receptors such as pregnane x receptor, termed PxR in rodents and SxR – steroid and xenobiotic receptor in humans. This receptor is considered a “master regulator” of defense against xenobiotics at the cellular and molecular levels. It regulates not only the human

MDR1 and P-gp, but the transcription of cytochrome P450 3A4 (CYP3A4) as well. Pregnane x receptor can be activated by naturally occurring steroids (pregnenolone and progesterone), synthetic glucocorticoids, and xenobiotics including dietary compounds, toxicants and drugs<sup>70,86,87</sup>. However, P-gp expression may be regulated not only by nuclear receptors like the PxR, but also by constitutive androstane receptor, and vitamin D binding receptor<sup>68</sup>.

In cell culture, expression of P-gp can be modulated by different conditions. Xenobiotics, environmental stress (heat and osmotic shock, low external pH arsenite and cadmium treatment), differentiating agents, and steroid hormones usually up-regulate P-gp levels. Other agents such as calcium channel blocker verapamil and dexamethasone have cell type-dependent effects<sup>71</sup>.

#### 1.5.5 Substrates, Inhibitors, and Enhancers of P-glycoprotein

A wide range of compounds interact and are under the influence of P-gp activity. Named as P-gp substrates, they do not necessarily share numerous common chemical features, but they do commonly display hydrophobic regions, positive charge at physiological pH, and they may contain aromatic rings<sup>71</sup>. P-gp substrates include anticancer, opioid, immunosuppressive and antifungal agents, antibiotics, steroids, beta- and calcium-channel blockers, toxic peptides (gramicidin D, valinomycin and N-caetyl-leucyl-leucyl-norleucinal [ALLN]), cardiac drugs (digoxin), fluorescent dyes (rhodamine 123), polycyclic aromatic hydrocarbons such as benzo(a)pyrene, and endogenous compounds (steroid opioids). The broad substrate specificity of P-gp may be due to multiple binding sites present in the P-gp (Table 1.1)<sup>66,67,70-72,85,88</sup>.

A Slightly controversial issue is that several substrates can also modulate P-gp function acting as inhibitors for MDR1/P-gp depending on their dose. Verapamil, cyclosporine, and quinidine act as substrates of P-gp at low doses, but at higher doses, they inhibit the P-gp potency<sup>85</sup>.

**Table 1.1 –Substrates and Inhibitors (\*) of P-glycoprotein and Their Therapeutic Use**

<b>Substrates</b>		<b>Inhibitors *</b>	
<b>Antibiotics</b>	<b>Ca<sup>+2</sup> Channel Blockers</b>	<b>Immunosuppressants</b>	<b>Antiarrhythmics</b>
Erythromycin *	<b>(Antiarrhythmics)</b>	Cyclosporin A *	Quinidine *
Tetracycline	Verapamil *	Tacromilus *	
Doxycycline	Digoxin	Vaspolar	<b>Natural Compounds</b>
Rifampin			Flavonoids *
Levofloxacin	<b>Chemotherapeutic</b>	<b>Opioids</b>	Coumarins *
Ofloxacin	Doxorubicin	Loperamide	Grapefruit juice *
	Daunorubicin	Morphine	Orange juice *
<b>Anticonvulsants</b>	Imatinib	Pentazocine	Capsaicin *
Phenytoin	Irinotecan	Methadone	Catechins (green tea) *
	Paclitaxel	Fentanyl	
<b>Antiemetics</b>	Vinblastine, Vincristine		
Domperidone	Actinomycin D	<b>Steroids</b>	
Ondansetron	Etoposide	Dexamethasone *	
		Aldosterone	
<b>Antifungal</b>	<b>Fluorescent Dyes</b>	Cortisol	
Itraconazole *	Rhodamine 123	Progesterone	
Ketoconazole *		Hydrocortisone	
		Corticosterone	
<b>β-Adrenoceptor Antagonists</b>	<b>H2-Antagonists</b>		
Bunitrolol	<b>Antiacids</b>	<b>Others</b>	
Celiprolol	Cimetidine	Ivermectin	
Tanilolol	Ranitidine		
Reserpine	<b>HIV Protease Inhibitors</b>		
	Amprenavir		
<b>Antihistaminic</b>	Indinavir *		
Chloropromazine	Saquinavir *		
Phenothiazine	Ritonavir *		

Common natural and synthetic compounds that are under influence of the P-gp efflux transport activity (substrates). Substrates that function also as inhibitors and specific inhibitors of P-gp activity are indicated with an \*. SOURCES: Matheny et al., 2001, Mealey, 2004, Cascorbi, 2006, Chan, 2008, Martinez et al., 2008, Zhou, 2008<sup>21,66,67,69,70,76</sup>.

Some naturally occurring substances like flavonoids found in fruits, vegetables and herbs, vitamin E and pharmaceutical excipients (tween 80, triton x-100, co-solvents) may act as P-gp inhibitors<sup>69</sup>. MDR1/P-gp inhibitors or modulators are pharmacologically effective agents able to reverse MDR in intact cells by reducing or blocking drug efflux activity of P-gp. Inhibitors block protein activity by three different mechanisms, binding non-competitively at the substrate-binding pocket to compete with other substrates, inhibiting ATP hydrolysis at the ATP binding site, or inhibiting protein kinase C which is involved with ATP coupling to P-gp. The ideal inhibitor is a non-competitive agent that binds allosterically to the protein and is irreversible regardless substrate or inhibitor concentration<sup>70,77</sup>.

Alternatively, expression of P-gp can be induced by chemical compounds such as rifampicin, paclitaxel, and reserpine, and by certain herbs and carotenoids that are able to activate one or more receptors for P-gp. Physical stress including x-irradiation, UV light irradiation, and heat shock are also described as potent enhancers for MDR1 and P-gp expression<sup>70</sup>.

#### **1.5.6 Relevance and Modulation of P-glycoprotein**

Disruption of P-gp function in mice demonstrated the relevance of P-gp on drug PK or disposition<sup>89-93</sup>. Elevated drug plasma levels, elevated drug concentrations in the brain, increased intestinal absorption, and reduced drug elimination was verified in *mdr1a* (-/-) knockout mice, homozygous for the genetic defect of P-gp<sup>69,92</sup>.

Also demonstrating the relevance of P-gp, loperamide is a classical example of P-gp drug transport with similar results in mice and people. As a potent antidiarrheal opioid drug, loperamide reduces gastrointestinal motility by its action at opioid receptors with no central opioid effects such as excitement, and respiratory depression at usual or even high doses. In

wild-type mice, loperamide does not cross the blood-brain barrier; however, clinical doses were lethal in knockout mice<sup>66,70,91,94</sup>. Inhibition of P-gp using the antiarrhythmic agent quinidine resulted in respiratory depression in people due to an increase of drug penetration into the brain<sup>94</sup>.

Lack of MDR1 gene, genetic polymorphism or gene mutation is not associated with phenotypic abnormalities. Its clinical relevance is more related to drug hypersensitivity or intoxication and increased susceptibility to certain diseases, including viral immunodeficiency syndrome (HIV), Parkinson's disease, multi-drug resistant epilepsy, renal carcinoma, and inflammatory bowel disease as found in humans<sup>66,69,89,95-97</sup>.

In veterinary species, mutation of MDR1 was first discovered in 1995 in a subpopulation of collie dogs which was then considered to be an "ivermectin-sensitive" breed<sup>98</sup>. It affects 30 to 40% of the collie breed population and several other breeds such as Shetland and English sheepdogs, Australian and German shepherds, Australian cattle dogs, border collies, and bearded collies. This genetic mutation generates stop codons resulting in the production of a nonfunctional fragment of P-gp. Dogs homozygous recessive for a four-base pair deletion in the MDR1 gene sequence exhibit dose-related toxicity to the macrocyclic lactone class of drugs such as ivermectin<sup>66,96,97,99</sup>. Mice naturally deficient in *mdr1a* are also extremely sensitive to the neurotoxic effects of ivermectin, resulting in high drug accumulation into the brain<sup>100</sup>. Due to the clinical importance of P-gp, a rapid PCR-based method for detection of specific MDR1 mutation was developed using a small amount of genomic DNA from blood cells and currently, a commercial DNA test is available through the Veterinary Clinical Pharmacology Laboratory (VCPL) at the Washington State University College of Veterinary Medicine<sup>69,99</sup>. Certainly, the

knowledge about individual MDR1 genetic defects or mutations improves the ability of veterinary clinicians to predict potential effectiveness or intoxication of drugs in animals.

## 1.6 ***In Vitro* Techniques to Study Protein-Mediated Drug Transport**

Animal models lacking drug transporters (knockout mice) and animals characterized by mutations in transporter genes are highly valuable tools to investigate *in vivo*, the role of intestinal efflux transporters on the absorption, bioavailability, distribution and elimination of therapeutic drugs. However, different *in vitro* models including cell culture and Ussing chambers have been used to study drug transport and predict intestinal absorption. Many models demonstrated to be reliable and predictive of *in vivo* protein transporter function.

### 1.6.1 **Cell Culture**

Tissue or cell culture is a very useful *in vitro* technique to investigate the activity of transporter proteins using primary cells or established cells (cell lines) to understand absorption of several therapeutic drugs in the whole organism or *in vivo*. Cultured cells, primary cells or cell lines should remain viable and must exhibit phenotypical and functional properties reminiscent to the normal epithelium to be used for these studies. However, this technique, like any *in vitro* study, has some limitations<sup>101</sup>.

Primary cells or primary culture are highly desirable for many biotechnological and clinical studies including protein-mediated drug transport; however, limited life span of cells is considered one of the major issues of this model. Primary culture of intestinal cells lasting more than 10 days is still difficult to achieve in *in vitro* biology<sup>102</sup>.

The success of primary cell culture depends on several factors including the characteristics of the tissue and cells of interest (type, source, aging), collection and processing time, technique for cell isolation (mechanical or enzymatic), and ideal conditions to maintain the



cultured cells (temperature, nutrients and medium, CO<sub>2</sub>). Disaggregation of cells is the first and most vital step in the culture of primary cells. Different types of cells are isolated depending on the technique applied and a mistake at this stage is irreversible<sup>103</sup>.

Overgrowth of stromal cells such as connective tissue fibroblasts is a relevant problem since they suppress and overcome the growth of any other cells in culture. In addition to the fact that epithelial cells lining the intestine are closely associated with fibroblasts, the selection or isolation of these non-target cells may be favored by some digestion enzymes, techniques for cell isolation, and high nutrition media with large quantities of fetal calf serum<sup>102-104</sup>.

After tissue dissociation, another challenge in preparing crypts and single intestinal epithelial cells is to provide a stable microenvironment promoting cell-to-cell interactions and allowing preservation or reestablishment of gap junction connectivity. Contamination is also one of the most difficult challenges especially to the culture of intestinal cells, considering this, a highly contaminated tissue<sup>102,103</sup>.

Several protocols for primary culture of epithelial cells mainly from the small intestine in different animals have been published<sup>101,105-107</sup>. However in horses, only cells cultured from fetal kidney and spleen have been established<sup>104</sup>.

In addition to primary cells, cell lines are permanent cultures with unlimited proliferation capacity also recognized as an appropriate *in vitro* model to study protein-mediated drug transport. Caco-2 is a human colon adenocarcinoma cell line that resembles small intestinal epithelial cells in morphology and expression of various marker enzymes and protein transporter comparable to those in human jejunum. It represents an established model for examining human small intestine transport and protein transporters such as P-gp<sup>108-111</sup>. A study showed the expression of P-gp in Caco-2 was decreased in the presence of budesonide in a dose-dependent

manner. However, this glucocorticoid steroid demonstrated to be a dose-dependent inducer of P-gp in LS180 cells. LS180 is an intestinal human colon adenocarcinoma cell line, a suitable model for intestinal gene induction, more appropriate to study induction of P-gp<sup>111</sup>.

Using LLC-PK1 (pig kidney epithelial cells) and human ABCB1-transfected LLC-PK1 cells, methadone was demonstrated to be a substrate of P-gp even at high or supra-therapeutic concentrations (for example 5,000 ng/mL) and this protein was suggested to have weak stereoselectivity in methadone transport<sup>112</sup>.

Equine intestinal cell culture would be an outstanding *in vitro* model to investigate the interaction between opioid drugs and the transmembrane efflux transporter P-gp to understand and possibly predict absorption of methadone *in vivo*. However, most of the described nontransformed mammalian intestinal epithelial cell models are of human or rodent origin<sup>101</sup>.

### 1.6.2 Ussing Chamber

The Ussing chamber is a technique invented by Hans Ussing to study molecular transport across epithelia in the 1950s<sup>113</sup>. It has been extensively used to investigate *in vitro* transport and metabolism and to predict *in vivo* oral drug absorption along the gastrointestinal tract<sup>114</sup>. In addition, this transport chamber tool is very useful to explore transmembrane protein transporters and study protein-mediated drug transport across mucosal membranes<sup>115</sup>.

The Ussing chamber system consists of two acrylic half-chambers between which small segments of tissue are mounted. Chambers are connected to an electrical circuit by calomel electrodes with Ag-AgCl bridges and connected to a “U” shaped reservoir by polyethylene tubing which maintains the temperature and a physiologic environment. This allows measurement of the short-circuit current as an indicator of net ion transport across membranes and the spontaneous potential difference as an indicator of mucosal integrity<sup>116</sup>.

Compared to the cell culture technique, Ussing chambers better estimate or predict drug absorption percentage *in vivo* from membrane permeability due to a more reliable and precise measurement of the permeability coefficient. This is the greatest advantage between these two *in vitro* models. Additionally, the Ussing chamber tool can better reproduce the *in vivo* environment in real time<sup>115</sup>.

To our knowledge, the absorption of methadone after oral drug administration has not been reported in horses using the Ussing chamber technique. Furthermore, Ussing chambers have not been used to evaluate the P-gp mediated transport of methadone across the equine intestinal mucosa.

### 1.7 Hypothesis and Objectives

We hypothesize that expression of P-gp in different tissues, especially the gastrointestinal (GI) tract of horses affects absorption and pharmacokinetics (PK) of methadone, decreasing drug bioavailability after oral administration.

These studies investigate through *in vivo* and *in vitro* studies, the oral PK of methadone in horses and determine the expression of the MDR1 gene and P-gp in equine oral, gastric, and intestinal mucosa. These studies also determine the role of the intestinal MDR1 gene and P-gp on flux and transport of methadone in the jejunum mucosa of horse as an indicator of *in vivo* drug absorption after oral administration of methadone in horses. In addition, the protein-mediated drug transport activity was investigated by modulation of the P-gp.

### 1.8 References

1. Thurmon, J. C., Tranquilli, W. J., Benson, G. J. (1996) *Lumb & Jones' veterinary anesthesia*. Baltimore, MD: Williams and Wilkins p.928.
2. Deshpande, M. A., Holden, R. R., Gilron, I. (2006) The impact of therapy on quality of life and mood in neuropathic pain: What is the effect of Pain reduction? *Anesthesia and Analgesia*. 102, 1473-1479.

3. Hellyer, P. W., Bai, L., Supon, J.; Quail, C., Wagner, A. E., Mama, K. R., Magnusson, K. R. (2003) Comparison of opioid and alpha-2 adrenergic receptor binding in horse and dog brain using radioligand autoradiography. *Veterinary Anaesthesia and Analgesia*. 30, 172-182.
4. Clarke, K. W., Paton, B. S. (1988) Combined use of detomidine with opiates in the horse. *Equine Veterinary Journal*. 20(1), 331-334.
5. Glowaski, M. M. (2007) Treating animal pain: The clinical perspective. *Veterinary Pharmacology 2007. American Academy of Veterinary Pharmacology and Therapeutics. 15<sup>th</sup> Biennial Symposium* Monterey, CA. May 20-24, 2007.
6. Driessen, B. (2008) Perioperative pain management in the horse: Can we effectively inhibit/prevent 'wind-up'? *The 14<sup>th</sup> Annual International Veterinary Emergency and Critical Care Symposium (IVECCS)*. Phoenix, AZ. September 17-21, 2008.
7. Skarda, R. T. (1996) Local and regional anesthetic and analgesic techniques: horses. In: Thurmon JC, Tranquilli WJ, Benson GJ, eds. *Lumb & Jones' veterinary anesthesia*. Baltimore, MD: Williams and Wilkins. p.448-478.
8. Natalini, C. C., Robinson, E. P. (2000) Evaluation of the analgesic effects of epidurally administered morphine, alfentanil, butorphanol, tramadol, and U50488H in horses. *American Journal of Veterinary Research*. 61(12), 1579-1586.
9. Robinson, E. P., Natalini, C. C. (2002) Epidural anesthesia and analgesia in horses. *The Veterinary Clinics of North America: Equine practice*. 18, 61-82.
10. Natalini, C. C., Robinson, E. P. (2003) Effects of epidural opioid analgesics on heart rate, arterial blood pressure, respiratory rate, body temperature, and behavior in horses. *Vet. Therapeutics*. 4(4), 364-375.
11. Natalini, C. C., Polydoro, A. S., Linardi, R. L. (2006) Analgesic effects of subarachnoidally administered hyperbaric opioids in horses. *American Journal of Veterinary Research*. 67(6), 941-946.
12. Clark, J. O., Clark, T. P. (1999) Analgesia. *Clinical Pharmacology and Therapeutics. Veterinary Clinics of North America: Equine Practice*. 15(3), 705-723.
13. Hall, L. W., Clarke, K. W., Trim, C. M. (2001) *Veterinary Anaesthesia* 10ed. W.B.Saunders. p.561.
14. Cohen, M. J., Schecter, W. P. (2005) Perioperative pain control: A strategy for management. *Surgical Clinics of America*. 85, 1243-1257.
15. Yoshimura, M., Furue, H. (2006) Mechanisms for the anti-nociceptive actions of the descending noradrenergic and serotonergic systems in the spinal cord. *Journal Pharmacol. Sci.* 101, 107-117.

16. Gourlay, G. K. (2005) Advances in opioid pharmacology. *Support Care Cancer*. 13, 153-159.
17. Combie, J., Dougherty, J., Nugent, C. E., Tobin, T. (1979) The pharmacology of narcotic analgesics in the horse. IV. Dose and time response relationships for behavioral responses to morphine, meperidine, pentazocine, anileridine, methadone, and hydromorphone. *J. Equine Med Surg*. 3, 377-385.
18. Centeno, C., Vara, F. (2005) Intermittent subcutaneous methadone administration in the management of cancer pain. *Journal of Pain & Palliative Care Pharmacotherapy*. 19(2), 7-12.
19. Molina, P.E. (2005) Opioids and opiates: analgesia with cardiovascular, haemodynamic and immune implications in critical illness. *Jornal of Internal Medicine*. 259, 138-154.
20. Lauretti, G. R. (2006) Highlights in opioid agonists and antagonists. *Expert Rev. Neurotherapeutics*. 6(4), 613-622.
21. Chan, L-N. (2008) Opioid analgesics and the gastrointestinal tract. *Practical Gastroenterology. Nutrition Issues in Gastroenterology*. Series 64-65, 37-50.
22. Dhawan, B. N., Cesselin, F., Raghubir, R., Reisine, T., Bradley, P. B., Portoghese, P. S., Hamon, M. (1996) International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacological Reviews*. 48(4), 567-592.
23. Sinatra, R. S. (2006) Peripherally acting Mu-opioid-receptor antagonists and the connection between postoperative ileus and pain management: The anesthesiologist's view and beyond. *Journal of PeriAnesthesia Nursing*. 21(2A), S16-S23.
24. Tobin, T. (1978) Narcotic analgesics and the opiate receptor in the horse. *J. Equine Med Surg*. 2, 397-399.
25. Tobin, T., Combie, J., Shults, T. (1979) Pharmacology review: actions of central stimulant drugs in the horse. II. *J. Equine Med Surg*. 3, 102-109.
26. Tobin, T., Combie, J., Shults, T., Dougherty, J. (1979) The pharmacology of narcotic analgesics in the horse. III. Characteristics of the locomotor effects of fentanyl and apomorphine. *J. Equine Med Surg*. 3, 284-288.
27. Page, G. G. (2005) Immunologic effects of opioids in the presence of absence of pain. *Journal of Pain and Symptom Management*. 29(5), 25-29.
28. Pippi, N. L., Lumb, W. V. (1979) Objective tests of analgesic drugs in ponies. *Am. J. Vet. Res*. 40(7), 1082-1086.
29. Muir, W. W., Skarda, R. T., Sheehan, W. C. (1978) Cardiopulmonary effects of narcotic agonists and a partial agonist in horses. *Am. J. Vet. Res*. 39(10), 1632-1635.

30. Pippi, N. L., Lumb, W. V., Fialho, S. A. G., Scott, R. J. (1979) A model of evaluation pain in ponies. *J. Eq. Med. Surg.* 3, 430-435.
31. Kamerling, S. (1988) Dose related effects of the kappa agonist U-50, 488H on behavior, nociception and autonomic response in the horse. *Equine Veterinary Journal.* 20(2), 114-118.
32. Hagedorn, H. W., Meiser, H., Zankl, H., Schulz, R. (1999) Methadone screening of racehorses. *Journal of Analytical Toxicology.* 23, 609-614.
33. Maxwell, L. K., Thomasy, S. M., Slovis, N., Kollias-Baker, C. (2003) Pharmacokinetics of fentanyl following intravenous and transdermal administration in horses. *Equine Veterinary Journal.* 35(5), 484-490.
34. Shir, Y., Shenkman, Z., Shavelson, V., Davidson, E., Rosen, G. (1998) Oral Methadone for the treatment of severe pain in hospitalized children: A report of five cases [Case Report]. *The Clinical Journal of Pain.* 14(4), 350-353.
35. Branson K. R., Gross M. E., Booth N. H. (1995) Opioid agonists and antagonists. IN: Adams, H. R. *Veterinary Pharmacology and Therapeutics.* 7ed. 274-310.
36. Rowlingson J. C., Murphy T. M. (2000) Chronic pain. IN: Miller R.D. *Anesthesia* 5ed. 2351-2379.
37. Dale, O., Hoffer, C., Sheffels, P., Kharasch, E. D. (2002) Disposition of nasal, intravenous, and oral methadone in healthy volunteers. *Clinical Pharmacology & Therapeutics.* 72, 536-545.
38. Eap, C. B., Buclin, T., Baumann, P. (2002) Interindividual Variability of the Clinical Pharmacokinetics of Methadone. *Clinical Pharmacokinetics.* 41(14), 1153-1193.
39. Bowsher, D. (2003) The treatment of neuropathic pain. IN: Bountra C., Munglani R., Schmidt W.K. *Pain.* 549-558.
40. Toombs, J. D., Kral, L. A. (2005) Methadone treatment for pain states. *American Family Physician.* 71(7), 1353-1358.
41. Wikipedia contributors, "Methadone," *Wikipedia, The Free Encyclopedia*, <http://en.wikipedia.org/w/index.php?title=Methadone&oldid=329778261> (accessed December 11, 2009).
42. Payte, J. T., Smith, J., Woods, J. (2001) Basic Pharmacology: How methadone works? The pharmacology of opioids. *National Alliance of Methadone Advocates, Educational Series*, number 5.2.
43. Lugo, R. A., Satterfield, K. L., Kern, S. E. (2005) Pharmacokinetics of methadone. *Journal of Pain & Palliative Care Pharmacotherapy.* 19, 13-24.

44. Inturrisi, C. E. (2005) Pharmacology of methadone and its isomers. *Minerva Anesthesiology*. 71, 435-437.
45. Shimoyama, N., Shimoyama, M., Elliot, K. J., Inturrisi, C. E. (1997) D-methadone is antinociceptive in the rat formalin test. *The Journal of Pharmacology and Experimental Therapeutics*. 283(2), 648-652.
46. Boulton, D. M., Arnaud, P., Devane, C. L. (2001) Pharmacokinetics and pharmacodynamics of methadone enantiomers after a single oral dose of racemate. *Clinical Pharmacology & Therapeutics*. 70(1), 48-57.
47. Chhabra, S., Bull, J. (2008) Methadone. *American Journal of Hospice and Palliative Medicine*. 25(2), 146-150.
48. Olbrich, V. H, Mosing, M. (2003) A comparison of the analgesic effects of caudal epidural methadone and lidocaine in the horse. *Vet. Anaesthesia and Analgesia*. 30(3):156-164.
49. Lotsch, J., Geisslinger, G. (2006) Current evidence for a genetic modulation of the response to analgesics. *Pain*. 121, 1-5.
50. Robertson, S. A., Taylor, P. M. (2004) Pain management in cats -past, present, and future. Part 2. Treatment of pain -clinical pharmacology. *Journal of Feline Medicine and Surgery*. 6, 321-333.
51. Kukanich, B., Lascelles, B. D., Aman, A. M., Mealey, K. L. & Papich, M. G. (2005) The effects of inhibiting cytochrome P450 3A, p-glycoprotein, and gastric acid secretion on the oral bioavailability of methadone in dogs. *Journal of Veterinary Pharmacology and Therapeutics*. 28, 461-466.
52. Kukanich, B., Borum, S. L. (2008) The disposition and behavioral effects of methadone in Greyhounds. *Veterinary Anaesthesia and Analgesia*. 35, 242-248.
53. Birkett, D. J. (2002) *Pharmacokinetics made easy*. McGraw-Hill, Sydney, NY. p.132.
54. Linardi, R.L., Stokes A.M., Barker S.A., Short C., Hosgood G., Natalini C.C. (2009) Pharmacokinetics of the injectable formulation of methadone hydrochloride administered orally in horses. *J. Vet. Pharmacol. Therap*. 32, 492-497.
55. Gourlay, G. K., Wilson, P. R., Glynn, C. J. (1982) Pharmacodynamics and pharmacokinetics of methadone during the perioperative period. *Anesthesiology*. 57, 458-467.
56. Gourlay, G. K., Willis, R. J., Lamberty, J. (1986) A double-blind comparison of the efficacy of methadone and morphine in postoperative pain control. *Anesthesiology*. 64, 322-327.
57. Clark, D. C. (2008) Understanding methadone metabolism. *Anesthesiology*. 108, 351-352.

58. Foster, D. J., Somogyi, A. A., Bochner, F. (1999) Methadone N-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4. *British Journal of Clinical Pharmacology*. 47, 403-412.
59. Oda, Y., Kharasch, E. D. (2001) Metabolism of methadone and levo-alpha-acethylmetadol (LAAM) by human intestinal cytochrome P450 3A4 (CYP3A4), potential contribution of intestinal metabolism to presystemic clearance and bioactivation. *The Journal of Pharmacology and Experimental Therapeutics*. 298, 1021-1032.
60. Abbott, F. S., Slatter, J. G., Burton, R., Kang, G. I. 1985 Methadone metabolism in the rat in vivo: identification of a novel formamide metabolite. *Xenobiotica*. 15(2), 129-140.
61. Neuhoﬀ, S (2005) Refine *in vitro* models for prediction of intestinal drug transport. Role of pH and extracellular additives in the Caco-2 cell model. Dissertation. *Acta Universitatis Upsaliensis*. p.84.
62. Olsen, L. (2007) Drugs in horses: Pharmacokinetics and Pharmacodynamics. *Doctoral Thesis. Swedish University of Agricultural Sciences*. p.56.
63. Curatolo, W. (1998) Physical chemical properties of oral drug candidates in the discovery and exploratory development settings. *PSTT*. 1(9), 387-393.
64. Kharasch, E. D., Hoffer, C., Whittington, D. (2004) The effect of quinidine, used as a probe for the involvement of p-glycoprotein, on the intestinal absorption and pharmacodynamics of methadone. *British Journal of Clinical Pharmacology*. 57, 600-610.
65. Di Pietro, A., Dayan, G., Conseil, G. et. al. (1999) P-glycoprotein-mediated resistance to chemotherapy in cancer cells: using recombinant cytosolic domains to establish structure-function relationships. *Brazilian Journal of Medical and Biological Research*. 32, 925-939.
66. Mealey, K.L. (2004) Therapeutic implications of the MDR-1 gene. *Journal of Veterinary Pharmacology and Therapeutics*. 27, 257-264.
67. Cascorbi, I. (2006) Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. *Pharmacology & Therapeutics*, 112, 547-473.
68. Marchetti, S., Mazzant, R., Beijnen, J.H., Schellens, J.H.M. (2007) Clinical relevance of drug-drug and herb-drug interactions mediated by the ABC Transporter ABCB1 (MDR1, P-glycoprotein). *The Oncologist*, 12, 927-941.
69. Martinez, M., Modric, S., Sharkey, M. et al. (2008) The pharmacogenomics of P-glycoprotein and its role in veterinary medicine. *Journal of Veterinary Pharmacology and Therapeutics*. 31, 285-300.
70. Zhou, S.F. (2008) Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*. 38(7-8), 802-832.



71. Fardel, O., Lecureur, V., Guillouzo, A. (1996) The P-glycoprotein multidrug transporter. *Gen. Pharmac.*, 27(8), 1283-1291.
72. Kimura, Y., Morita, S., Matsuo, M., Ueda, K. (2007) Mechanism of multidrug recognition by MDR1/ABCB1. *Cancer Science*. 98(9), 1303-1310.
73. Alexandrova, R. (1998) Multidrug resistance and P-glycoprotein. *Experimental Pathology and Parasitology*, 62-65.
74. Gottesman, M.M., Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.*, 62, 385-427.
75. Juliano, R.L., Ling, V. (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta*, Amsterdam. 455, 152-162.
76. Matheny, C.J., Lamb, M.W., Brouwer, K.L.R., Pollack, G.M. (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy*, 21(7), 778-796.
77. Sharom, F.J. (2006) Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABCB1). *Biochemistry and Cell Biology*. 84, 979-992.
78. Clarke D.M. 2006 Structure and function of membrane transport proteins. <http://biochemistry.utoronto.ca/clarke/bch.html>. Accessed on Dec. 2009.
79. Thompson, S.J., Koszdin, K., Bernards, C.B. (2000) Opioid-induced analgesia is increased and prolonged in mice lacking P-glycoprotein. *Anesthesiology*. 92(5), 1392-1399.
80. Wang, J.S., Yiang, R., Taylor, R.M. et al. (2004) Brain penetration of methadone (R)- and (S)-enantiomers is greatly increased by P-gp deficiency in the blood-brain barrier of Abcb1a gene knockout mice. *Psychopharmacologia*. 173(1), 132-138.
81. Zimmermann, C., Gutmann, H., Hruz, P. et al. (2005) Mapping of multidrug resistance gene 1 and multidrug resistance associated protein isoform 1 to 5 mRNA expression along the human intestinal tract. *The American Society for Pharmacology and Experimental Therapeutics*. 33(2), 219-224.
82. Lotsch, J., Sharke, C., Tegeder, I., Geisslinger, G. (2002) Drug Interactions with patient-controlled analgesia. *Clinical Pharmacokinetics*. 41(1), 31-57.
83. Katsura, T., Inui, K-i. (2003) Intestinal absorption of drugs mediated by drug transporters: Mechanisms and Regulation. *Drug Metab. Pharmacokin.* 18(1), 1-15.
84. Sun, J., He, Z.G., Chen, G. Wang, S. J., Hao, X. H., Zou, M., J. (2004) Multidrug resistance P-glycoprotein: crucial significance in drug disposition and interaction. *Medical Science Monitor*. 10(1), RA5-14.

85. Fromm, M.F. (2004) Importance of P-glycoprotein at blood-tissue barriers. *TRENDS in Pharmacological Sciences*. 25(8), 423-429.
86. Loscher, W., Potschka, H. (2005) Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Progress in Neurobiology*. 76(1), 22-76.
87. Miller, D. S., Bauer, B., Hartz, A. M. S. (2008) Modulation of P-glycoprotein at the blood-brain barrier: Opportunities to improve central nervous system pharmacotherapy. *Pharmacological Reviews*. 60(2), 196-209.
88. Takano, M., Yumoto, R., Murakami, T. (2006) Expression and function of efflux drug transporters in the intestine. *Pharmacology and Therapeutics*. 109, 137-161.
89. Mukhopadhyay, T., Batsakis, J. G., Kuo, M. T. (1988) Expression of the *mdr* (P-glycoprotein) gene in Chinese hamster digestive tracts. *Journal of the National Cancer Institute*. 80(4), 269-275.
90. Collaghan, R., Riordan, J. R. (1993) Synthetic and natural opiates interact with P-glycoprotein in multidrug-resistant cells. *The Journal Biological Chemistry*. 268(21), 16050-16064.
91. Schinkel A.H, Wagenaar E., Mol C.A.A.M., Deemter L.V. (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation*. 97, 2517-2524.
92. Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A. H., Smit, J. W., Meijer, D. K. F., Borst, P., Nooijen, W. J., Beijnen, J. H., van Tellingen, O. (1997) Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proceedings of the National Academy of Sciences of the United States of America*. 94(5), 2031-2035.
93. Bouer, R., Barthe, L., Philibert, C., Tournaire, C., Woodley, J., Houin, G. (1999) The roles of P-glycoprotein and intracellular metabolism in the intestinal absorption of methadone: in vitro studies using the rat everted intestinal sac. *Fundamental and Clinical Pharmacology*. 13(4), 494-500.
94. Sadeque A. J. M., Wandel C., He, H. et al. (2000) Increased drug delivery to the brain by glycoprotein inhibition. *Clinical Pharmacology and Therapeutics*. 68(3), 231-237.
95. Wilk, J. N., Bilsborough, J., Viney, J. L. (2005) The *mdr1a*<sup>-/-</sup> mouse model of spontaneous colitis: a relevant and appropriate animal model to study inflammatory bowel disease. *Immunol. Res*. 31(2), 151-160.
96. Mealey, K. L., Bentjen, S. A., Gay, J. M., Cantor, G. H. (2001) Ivermectin sensitivity in collies is associated with a deletion mutation of the *mdr1* gene. *Pharmacogenetics*. 11(8), 727-733.

97. Mealey, K. L., Munyard, K. A., Bentjen, S. A. (2005) Frequency of the mutant MDR1 allele associated with multidrug sensitivity in a sample of herding breed dogs living in Australia. *Veterinary Parasitology*. 131(3-4), 193-196.
98. Pulliam, J. D., Seward, R. L., Henry, R. T., Steinberg, S. A. (1985) Investigating ivermectin toxicity in Collies. *Veterinary Medicine*. 80(6), 33-40.
99. Geyer, J., Doring, B., Godoy, J. R., et al. (2005) Development of a PCR-based diagnostic test detecting a nt230(del4) MDR1 mutation in dogs: verification in a moxidectin-sensitive Australian Shepherd. *Journal of Veterinary Pharmacology and Therapeutics*. 28, 95-99.
100. Lankas, G. R., Cartwright, M. E., Umbenhauer, D. (1997) P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicology and Applied Pharmacology*. 143(2), 357-365.
101. Golaz, J. L., Vonlaufen, N., Hemphill, A., Burgener, I. A. (2007) Establishment and characterization of a primary canine duodenal epithelial cell culture. *In Vitro Cell. Dev. Biol.-Animal*. 43, 176-185.
102. Kaeffer, B. (2002) Mammalian intestinal epithelial cells in primary culture: A mini-review. *In Vitro Cell. Dev. Biol.-Animal*. 38, 123-134.
103. Freshney, R. I. (2005) Culture of Animal Cells: A Manual of Basic Technique. Fifth Edition. John Wiley & Sons, Hoboken, NJ, USA. p.642.
104. Watanabe, S., Ishikawa, Y., Hara, H., Hanzawa, K., Mukoyama, H. (1997) A method of primary cell culture for establishing equine long-term culture cell lines. *J. Equine Sci.* 8(4), 95-99.
105. Evans, G. S., Flint, N., Somers, A. S., Eyden, B., Potten, C. S. (1992) The development of a method for the preparation of rat intestinal epithelial cell primary cultures. *Journal of Cell Science*. 101, 219-231.
106. Utoguchi, N., Watanabe, Y., Suzuki, T., Maehara, J., Matsumoto, Y., Matsumoto, M. (1997) Carrier-mediated transport of monocarboxylic acids in primary cultured epithelial cells from rabbit oral mucosa. *Pharmaceutical Research*. 14(3), 320-324.
107. Macartney, K. K., Baumgart, D. C., Carding, S. R., Brubaker, J. O., Offit, P. A. (2000) Primary murine small intestinal epithelial cells, maintained in long-term culture, are susceptible to rotavirus infection. *Journal of Virology*. 74(12), 5597-5603.
108. Maier, A., Zimmermann, C., Beglinger, C., Drewe, J., Gutmann, H. (2007) Effects of budesonide on P-glycoprotein expression in intestinal cell lines. *British Journal of Pharmacology*. 150, 361-368.

109. Stormer, E., von Moltke, L. L., Perloff, M. D., Greenblatt, D. J. (2001) P-glycoprotein interactions of nefazodone and trazodone in cell culture. *Journal Clin. Pharmacol.* 41, 708-714.
110. Schrickx, J., Fink-Gremmels, J. (2007) P-glycoprotein-mediated transport of oxytetracycline in the Caco-2 cell model. *J. Vet. Pharmacol. Therap.* 30, 25-31.
111. Li, Q., Sai, Y., Kato, Y., Tamai, I., Tsuji, A. (2003) Influence of drugs and nutrients on transporter gene expression levels in Caco-2 and LS180 intestinal epithelial cell lines. *Pharmacoeutical Research.* 20(8), 1119-1124.
112. Crettol, S., Digon, P., Golay, K. P., Brawand, M., Eap, C. B. (2007) In vitro P-glycoprotein-mediated transport of (R)-, (S)-, (R,S)-methadone, LAAM and their main metabolites. *Pharmacology.* 80, 304-311.
113. Li, H., Sheppard, D. N., Hug, M. J. (2004) Transepithelial electrical measurements with the Ussing chamber. *J. Cystic Fibrosis.* 3, suppl.2, 123-126.
114. Lennernas, H. (2007) Animal data: The contributions of the Ussing chamber and perfusion systems to predicting human oral drug delivery *in vivo*. *Advanced Drug Deliverd Reviews.* 59, 1103-1120.
115. Gotoh, Y., Kamada, N., Momose, D. (2005) The advantages of Ussing chamber in drug absorption studies. *Journal of Biomolecular Screening.* 10(5), 517-523.
116. Brown, D.R., O'Grady, S.M. (2008) The Ussing chamber and measurement of drug actions on mucosal ion transport. *Current Protocols in Pharmacology.* Unit 7.12.1-7.12.17 Supplement.

**CHAPTER 2.**  
**PHARMACOKINETICS OF THE INJECTABLE FORMULATION OF METHADONE**  
**HYDROCHLORIDE ADMINISTERED ORALLY IN HORSES \***

---

\* With permission from the *Journal of Veterinary Pharmacology and Therapeutics*

## 2.1. Introduction

Methadone hydrochloride [6-(dimethylamino)-4,4-diphenyl-3-heptanone hydrochloride molecular formula of  $C_{12}H_{27}NO.HCl$ , molecular weight 345.91; AAI Pharma Inc., Charleston, SC, USA] is a synthetic  $\mu$ -opioid receptor agonist. Dissimilar in structure yet nearly equipotent to morphine, methadone possesses distinct properties that distinguish it from all other opioids, which make it an excellent analgesic. Characteristics of methadone that enhance its analgesic properties include its nonopioid actions of noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist and serotonin- and norepinephrine-reuptake inhibition<sup>1,2</sup>. Blocking the NMDA receptor may prevent the development of tolerance to methadone as receptor activation can produce central sensitization, which lowers central nervous system (CNS) pain thresholds<sup>3</sup>. Methadone is commonly used as a racemic mixture of two enantiomers, (R-) or levo- (l-) and (S-) or dextro-(d-) isomers<sup>1,4</sup>, which have different pharmacologic activities. The R-form accounts for most of the opioid effect and is considered 50 times more potent than the S-form; however, both enantiomers have affinity for the NMDA receptor<sup>5</sup>.

The pharmacokinetic (PK) characteristics of oral methadone have been investigated in people<sup>1,2,6</sup>, cats<sup>7</sup> and dogs<sup>8,9</sup>. In people, the PK profile of methadone is characterized by rapid absorption with high oral bioavailability (70–80%), high protein binding (87%), long elimination half-life (20–35 h) and wide interindividual variability. Methadone is well distributed throughout the body and may accumulate in tissues after multiple doses, contributing to its long half-life. It undergoes extensive stereoselective hepatic metabolism and renal elimination. Cytochrome P450 (CYP) 3A4 is the major enzyme involved in the biotransformation of methadone enantiomers by N-demethylation, producing 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) as the main therapeutically inactive metabolite

in people<sup>1,2,6</sup>. Some studies suggest that CYP2B6, CYP2D6 and other enzymes may also contribute to metabolism of methadone, but to a lesser extent<sup>2,6,10,11</sup>. In dogs, the metabolism of methadone is still not fully characterized. Unlike in humans, EDDP and EDMP are considered minor metabolites and the CYP3A4 may not be the major metabolizing enzyme in dogs. The renal elimination of intact methadone and its metabolites accounts for approximately 20–50% of total excretion, and is directly dependent on urine pH<sup>9</sup>.

The systemic use of opioids in horses is limited because of the risk of sympathetic stimulation and CNS excitation<sup>12-14</sup>. Local administration has been examined to determine the duration and effectiveness of analgesia with minimal opioid-induced adverse effects; however, limited analgesic effects have been demonstrated when opioids are locally administered, such as by the epidural and subarachnoid routes<sup>15-19</sup>. So far, there are no studies describing the PK of oral opioids in horses. This study describes the PK of methadone hydrochloride (HCl) in 12 adult healthy horses after administration of single oral doses (0.1, 0.2 or 0.4 mg/kg). The physical effects were also observed and recorded.

## **2.2 Materials and Methods**

### **2.2.1 Animals**

This study was approved by the Louisiana State University Institutional Animal Care and Use Committee. Twelve healthy adult horses (six gelding and five female Thoroughbreds and one female Quarter horse) with mean (SD) body weight of 498.5 kg (42.2) and mean (SD) age of 4.7 years (2.4) were evaluated. The horses did not receive medications for at least 4 weeks prior to the study. Horses were placed in stalls two days before the study and received both complete pellet ration and grass hay twice daily, with free access to water. Horses were fasted for 12 hours before drug administration with continuous access to water. A 14-gauge catheter (B.D.

Angiocath i.v. catheter, 14-gauge x5.25 inch catheter; Becton Dickinson Infusion Therapy System Inc., Franklin Lakes, NJ, USA) was placed in the left jugular vein for blood collection.

### **2.2.2 Study Design**

Horses were randomly placed into three groups to receive a single dose of methadone HCl (methadone hydrochloride injection, USP 10 mg/mL, *aaiPharma*, Wilmington, NC, USA) administered directly into their mouth using a 60cc syringe containing 30 mL of commercial corn syrup (0.1, 0.2, or 0.4 mg/kg, intravenous formulation, 4 horses/dose). Blood samples (10 mL) were collected into sterile vacutainer blood collection tubes, no additive (BD Franklin Lakes, NJ, USA). They remained at room temperature for 30–60 minutes, and were then centrifuged at 2808 g for 10 min. Serum was transferred to propylene sterile tubes (Biomed Resource Inc., Torrance, CA, USA) and stored at -20°C until analysis. Horses were subjectively evaluated for physical effects. Horses were observed for locomotor activity, excitation or sedation (low head position). Cardiac and respiratory rates (auscultation and respiratory movement count) were recorded. The presence of gastrointestinal (GI) motility was also evaluated, through 4 quadrant auscultation.

### **2.2.3 Serum Sampling and Clinical Evaluation**

Blood was collected from the jugular vein prior to (0) and at 15 and 30 minutes, 1, 2, 3, 4, 5, 6, 9, 12, and 24 h after drug administration. Clinical evaluation was performed at the same time points.

### **2.2.4 Serum Analysis**

Methadone concentrations in equine serum samples were determined using a gas chromatography/mass spectrometry (GC/MS) instrument (Agilent Technologies, Wilmington,



DE; 6890 Series GC System with a 5973 MS Detector) operating in the positive ion, electron impact ionization mode.

### 2.2.5 Chemicals

DL-Methadone-*d*<sub>3</sub> (1 mg/ml in methanol, Isotec Inc., Miamisburg, OH, USA) was used as an internal standard and Methadone hydrochloride (1.13 mg – equivalent to 1.01 mg free base/ml GC grade methanol solution, Sigma, St. Louis, MO, USA), water, methanol, and methylene chloride (all Optima grade, Fisher Scientific, Fair Lawn, NJ, USA), and Toxi-Lab Toxi-Tubes-A extraction tubes (Toxi-Lab Toxi-Tubes-A extraction tubes Varian, Inc., Lake Forest, CA, USA) were also used for the analyses.

### 2.2.6 Sample Preparation

Serum samples (1.0 mL) were aliquotted along with a method blank (1.0 mL Optima grade water) and serum blanks (with and without internal standard) into separate tubes. Methadone working standards were prepared by diluting the internal standard in methanol to concentrations of 0.1, 1 and 10 µg/mL. Calibration standards were prepared in drug free equine serum by diluting the working standard into 1.0 ml of serum to concentrations of 2, 5, 10, 20, 50, 100, 250 and 500 ng/mL. The internal standard (methadone-*d*<sub>3</sub> – 50 ng) was added to 1 mL aliquots of each control and test sample (except as indicated). Optima grade water was added to bring the samples up to a final volume of 5.0 mL. The samples were transferred to Toxi-Tubes for extraction following the manufacturer's instructions. Extracted samples were centrifuged; the supernatant was transferred to a clean tube and evaporated in a water bath at 45 °C under a continuous stream of dry nitrogen (N-EVAP, Organization Associates, South Berlin, MA, USA). The resulting residue was dissolved in 150 µL of methylene chloride and transferred to a micro-injection vial for GC/MS analysis.

### 2.2.7 Instrumentation

A DB-5 column (30 m x 0.25 mm I.D., film thickness 0.25  $\mu$ m, Agilent – J&W Scientific, Santa Clara, CA, USA) was used for GC/MS analysis. The oven temperature was programmed with continuous carrier gas (helium) flow (1 mL/min) and was operated using a temperature program; 100°C for 1 min rising up to 300 °C at 20 °C/min. The temperature was set at 250 °C for the injection port and 300°C for the transfer line. The mass spectra for methadone and methadone-*d*<sub>3</sub> were determined and fragment ions at *m/z* 294, 223 and 309 were used for the qualitative identification of methadone and 297, 226 and 312 *m/z* for the qualitative identification of methadone-*d*<sub>3</sub>. Concentrations were determined by producing a calibration curve using the peak area ratios of the analyte (methadone, 294 *m/z*) to the internal standard (methadone-*d*<sub>3</sub>, 297 *m/z*). Chromatographic data were processed using AGILENT software (Agilent ChemStation). The limit of quantification (LOQ) was defined as the lowest concentration that had a precision varying less than  $\pm 20\%$  (LOQ: 2 ng/mL). Inter- and intra-assay rate of eliminations of variation were 2.00–4.22% and 1.46–1.56%, respectively. The minimal acceptable correlation ( $R^2$ ) was 0.9989 for standard curves. Mean  $R^2$  was  $0.999 \pm 0.0003$ . This analysis was performed by the Louisiana State University Analytical System Laboratory, School of Veterinary Medicine.

### 2.2.8 Pharmacokinetics Analysis

A noncompartmental model was fitted to serum concentration data of each horse. Noncompartmental PK parameters were determined using the linear trapezoidal model with linear interpolation. Analysis was performed using the WINNONLIN computer software Version 5.1 (Pharsight Corporation, Mountain View, CA, USA). The first order rate constant associated with the terminal (log-linear) portion of the curve ( $\lambda_z$ ) was estimated by a linear regression

analysis using up to 9 serum log concentration-time points. The estimated terminal or elimination half-life ( $t_{1/2}$ ) was calculated using the equation<sup>20</sup>:

$$t_{1/2} = -\ln 2 / \lambda_z$$

The area under the concentration-time curve from 0 to last time point ( $AUC_{0-t}$ ) was estimated using the trapezoidal method. The mean residence time ( $MRT_{0-INF}$ ) was estimated by use of the equation of noninfusion models<sup>21</sup>:

$$MRT = AUMC_{0-INF} / AUC_{0-INF}$$

Estimated systemic or total body clearance ( $Cl$ ) was calculated as<sup>20</sup>:

$$Cl = \text{Dose} / AUC_{0-t}$$

Estimated apparent volume of distribution ( $V_d$ ) was calculated as<sup>22</sup>:

$$V_d = t_{1/2} \cdot Cl / 0.693$$

Clearance and volume of distribution were reported as  $Cl/F$  and  $V_d/F$ , respectively, as the oral bioavailability ( $F$ ) was not estimated in this study.

The estimated maximum serum drug concentration ( $C_{max}$ ) and time to maximum concentration ( $T_{max}$ ) were determined directly from the estimated concentration–time curves obtained for different doses.  $C_{max}/\text{dose}$  and  $AUC_{0-t}/\text{dose}$  (dose normalization) were also calculated.

### 2.2.9 Statistical Analysis

Statistical analysis was performed using SAS 9.1 (SAS Institute, Cary, NC, USA). Parameters with nonnormal distribution ( $MRT$ ,  $T_{max}$ , and  $AUC/\text{dose}$ ) were log transformed and statistical differences between the PK parameters ( $AUC$ ,  $MRT$ ,  $t_{1/2}$ ,  $Cl/F$ ,  $V_d/F$ ,  $C_{max}$ ,  $T_{max}$ ,  $AUC/\text{dose}$ ,  $C_{max}/\text{dose}$ ) were analyzed using one-way analysis of variance (ANOVA). *Ad hoc* comparison was made by Tukey's test with significance set at  $P < 0.05$ .

### 2.3 Results

No drug-induced adverse effects were observed in these horses for 24 h after administration of any of the 3 doses of methadone. Horses did not demonstrate signs of excitement, sedation, or increased locomotor activity. Physiological parameters for cardiac and respiratory rates were considered in the normal range, and no change was observed regarding the intestinal motility in any of the 4 quadrants after drug administration.

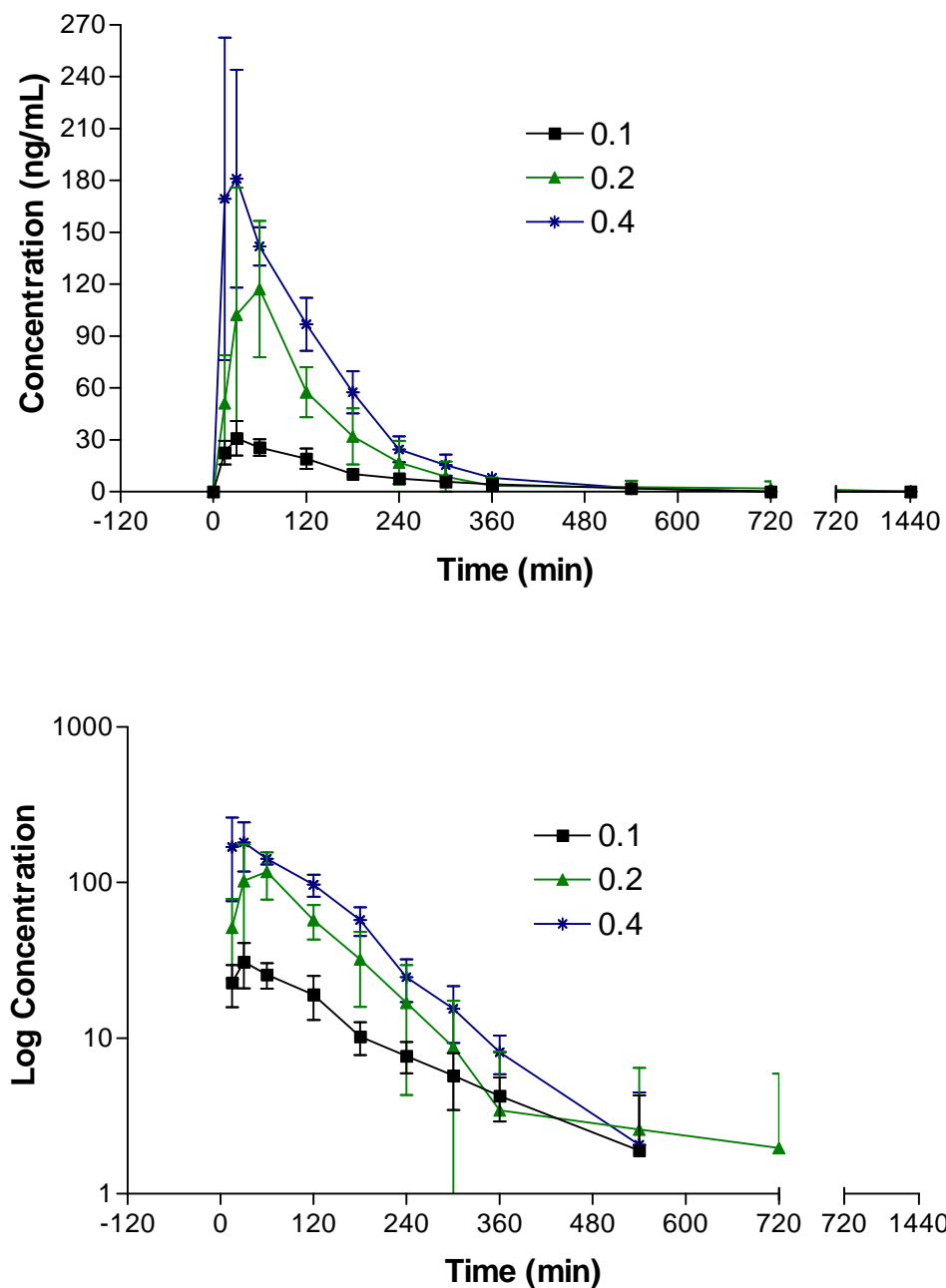
Serum methadone concentrations were detected in horses for 12 h after drug administration. The estimated serum concentration–time curves for all three doses of methadone were characterized by a biphasic profile with rapid absorption and elimination phases describing a first order process (Figure 2.1).

All serum methadone concentrations measured at specific time points were used for PK parameter calculation except for the 30 min sample of one horse receiving the dose of 0.2 mg/kg, which was unusable.

The estimated clearance ( $Cl/F$ ),  $MRT$ , estimated elimination half-life ( $t_{1/2}$ ), and estimated  $T_{max}$  were not significantly different across doses. Estimated area under the curve ( $AUC$ ), volume of distribution ( $V_d/F$ ), and  $C_{max}$  were significantly different across doses ( $P < 0.05$ ) (Table 2.1).

### 2.4 Discussion

Oral methadone was well tolerated in horses and none of the horses became excited or showed increased locomotor activity or physiologic alterations including decrease of respiratory rate, increase of cardiac rate, or decrease in intestinal motility at any dose.



**Figure 2.1 – Serum Concentration vs. Time Curves for Oral Administration of Methadone in Horses.**

Mean ( $\pm$ SD) serum methadone concentration from 12 healthy horses over 24 h following oral administration of methadone at doses of 0.1, 0.2 and 0.4 mg/kg body weight ( $n = 4$  each dose). Methadone concentration is in ng/mL (a) and using natural log (b). Points are connected by a line for better visualization and (SD) are represented as upper and lower bars.

**Table 2.1 – Pharmacokinetic Parameters of Oral Administration of Methadone**

Pharmacokinetic parameters										
Dose (mg/kg)	$AUC_{0-t}$ (ng.min/mL)		$MRT_{0-INF}$ (min)		$t_{1/2}$ (h)		$CL/F$ (mL/min/kg)		$V_d/F$	(L/kg)
	Mean	Median	Mean	Median	Mean	Median	Mean	Median	Mean	Median
0.1	5612 (1224.3) a	5405 (4809-6415)	203.1 (54.9) a	204.4 (167-239)	2.2 (35.6) a	2.2 (1.8-2.6)	17.3 (3.5) a	17.9 (14.8-19.8)	3.1 (0.7) a	3.2 (2.6-3.7)
0.2	17340 (8803.6) ab	14618 (11564-23115)	129.9 (48.8) a	112.8 (101-159)	1.3 (46.1) a	1 (0.7-1.8)	13.5 (5.2) a	13.7 (9.6-17.3)	1.2 (0.2) b	1.3 (1.1-1.4)
0.4	26028 (5474.6) b	25054 (21582-30474)	122.4 (7.0) a	122.6 (116-128)	1.5 (40.8) a	1.3 (1.1-2.0)	15.8 (3.2) a	16.1 (13.5-18.5)	2 (0.7) ab	1.7 (1.5-2.5)

Pharmacokinetic parameters								
Dose (mg/kg)	$C_{max}$ (ng/mL)		$T_{max}$ (min)		$AUC_{0-t}/dose$ (ng.min/mL)		$C_{max}/dose$ (ng/mL)	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median
0.1	33.9 (6.7) a	34.4 (28.2-39.5)	22.5 (8.6) a	22.5 (15-30)	56124 (12243) a	54050 (48093-64155)	338.6 (67.2) a	344.1 (282.1-395.1)
0.2	127.9 (36.0) b	124 (98.9-157)	37.5 (15.0) a	30 (30-45)	86698 (44018) a	73092 (57820-115576)	639.7 (178.0) b	620.1 (494.5-784.9)
0.4	193.5 (65.8) b	176.1 (145.6-241.3)	30 (21.2) a	22.5 (15-45)	65070 (13687) a	62637 (53955-76185)	483.7 (164.6) ab	440.3 (364.1-603.3)

Mean ( $\pm$ SD) and median (interquantiles) of the estimated pharmacokinetic parameters of oral administration of single doses of methadone (0.1, 0.2 and 0.4 mg/kg) to 12 healthy horses (n = 4 each dose). Significant differences are indicated by different letters ( $P < 0.05$ ).

The disposition of methadone following a single oral administration in horses was assumed as first order elimination and it was characterized by a biphasic serum profile with rapid absorption and elimination phases. Methadone disposition was considered a log-linear process as no drug saturation was observed even after administration of the highest dose of 0.4 mg/kg. Both compartmental and noncompartmental models are used to describe the disposition of methadone after oral or IV administration in people<sup>2</sup> and dogs<sup>8</sup>. In this study, the estimated pharmacokinetic parameters of methadone were determined by using a noncompartmental approach. As described for other species<sup>1,2,6,8,9</sup>, there was some individual variability among the horses regarding the PK parameters estimated.

Although oral bioavailability was not estimated as part of this study, methadone was rapidly absorbed and became systemically available after oral administration in horses with initial detection in the serum at the 15-min time point. In humans, orally administered methadone is usually measurable in plasma within 15–45 min. The estimated  $T_{\max}$  of oral methadone was faster in horses compared with people, which occurs at 2.5–4 h. Although the serum methadone concentrations associated with analgesia (therapeutic or effective concentration) are still undetermined for horses, the estimated  $C_{\max}$  was above the effective plasma concentration for humans, which ranges from 33 to 59 ng/mL<sup>23-25</sup>.

As the basic pH of the oral cavity is favorable to the absorption of alkaline drugs, we assume that at least part of methadone absorption takes place in the oral cavity through the local vasculature of the oral mucosa before the drug reaches and can be absorbed by the small intestine, considered to be the major site of drug absorption. A recent case report demonstrated long-lasting analgesic effects of buprenorphine after sublingual administration in horses, without signs of CNS excitement<sup>26</sup>. Most of the studies in humans refer to sublingual venous drainage as

the only site of absorption for oral opioids; however, an exact placement of opioid drug beneath the tongue in horses may not be required, considering that the oral mucosal sites differ between species in terms of anatomy and permeability<sup>26</sup>. One of the greatest advantages of oral administration and oral cavity absorption is the prevention of first pass hepatic metabolism by allowing absorption through gastrointestinal tract segments which are not drained by the portal vein<sup>27</sup>.

The oral PK of methadone in horses, characterized by short  $t_{1/2}$ , high  $Cl/F$  and small  $V_d/F$ , is similar to the PK reported in dogs<sup>8,9</sup>. It can possibly be explained by the single dose administered and rapid elimination of the drug. High  $Cl/F$  values indicate rapid drug elimination by the body, in agreement with the short  $t_{1/2}$ . The  $MRT$  also points to rapid drug elimination, and combined with other parameters, the small  $V_d/F$  may indicate restricted drug distribution. However, in people, the long  $t_{1/2}$  ( $> 20$  h) described for methadone could be related to the accumulation of drug in tissue binding sites with slow release back into plasma, especially after subsequent administrations or multiple dosing<sup>23-25</sup>.

These findings support our hypotheses that methadone is absorbed following oral administration in horses and reaches the therapeutic concentration reported in humans and other species without physical adverse effects. However, further investigations are necessary to determine the bioavailability of oral administration in horses before oral opioids can be considered as analgesic medication for the horse.

## 2.5 References

1. Eap, C.B., Buclin, T. & Baumann, P. (2005) Individual variability of the clinical pharmacokinetics of methadone. Implications for the treatment of opioid dependence. *Clinical Pharmacokinetics*, 41, 1153–1193.
2. Lugo, R.A., Satterfield, K.L. & Kern, S.E. (2005) Pharmacokinetics of methadone. *Journal of Pain & Palliative Care Pharmacotherapy*, 19, 13–24.



3. Hewitt, D.J. (2000) The use of the NMDA-receptor antagonists in the treatment of chronic pain. *The Clinical Journal of Pain*, 16, S73–S79.
4. Inturrisi, C.E. (2005) Pharmacology of methadone and its isomers. *Minerva Anesthesiology*, 71, 435–437.
5. Shimoyama, N., Shimoyama, M., Elliott, K.J. & Intrussi, C.E. (1997) *d*-Methadone is Antinociceptive in the Rat Formalin Test. *The Journal of Pharmacology and Experimental Therapeutics*, 283, 648–652.
6. Boulton, D.W., Arnaud, P. & DeVane, C.L. (2001) Pharmacokinetics and pharmacodynamics of methadone enantiomers after a single oral dose of racemate. *Clinical Pharmacology and Therapeutics*, 70, 48–57.
7. Robertson, S.A. & Taylor, P.M. (2004) Pain management in cats -past, present, and future. Part 2. Treatment of pain -clinical pharmacology. *Journal of Feline Medicine and Surgery*, 6, 321–333.
8. Kukanich, B., Lascelles, B.D., Aman, A.M., Mealey, K.L. & Papich, M.G. (2005) The effects of inhibiting cytochrome P450 3A, p-glycoprotein, and gastric acid secretion on the oral bioavailability of methadone in dogs. *Journal of Veterinary Pharmacology and Therapeutics*, 28, 461–466.
9. Kukanich, B. & Borum, S.L. (2008) The disposition and behavioral effects of methadone in Greyhounds. *Veterinary Anaesthesia and Analgesia*, 35, 242–8.
10. Foster, D.J., Somogyi, A.A. & Bochner, F. (1999) Methadone N-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4. *British Journal of Clinical Pharmacology*, 47, 403–412.
11. Oda, Y. & Kharasch, E.D. (2001) Metabolism of methadone and levo-alpha-acethylmetadol (LAAM) by human intestinal cytochrome P450 3A4 (CYP3A4), potential contribution of intestinal metabolism to presystemic clearance and bioactivation. *The Journal of Pharmacology and Experimental Therapeutics*, 298, 1021–1032.
12. Muir, W.W., Skarda, R.T. & Sheehan, W.C. (1978) Cardiopulmonary effects of narcotic agonists and a partial agonist in horses. *American Journal of Veterinary Research*, 39, 1632–1635.
13. Tobin, T. (1981) Horses running happy: fentanyl, morphine, and the other narcotic analgesics. In *Drugs and the Performance Horse*. Ed. Tobin, T., pp. 199–215. Charles C. Thomas Publisher, Springfield.
14. Kamerling, S. (1988) Dose related effects of the kappa agonist U-50, 488H on behavior, nociception and autonomic response in the horse. *Equine Veterinary Journal*, 20, 114–118.

15. Skarda, R.T. (1996) Local and regional anesthetic and analgesic techniques: horses. In *Lumb & Jones' Veterinary Anesthesia* 3<sup>rd</sup> edn. Eds Thurmon, J.C., Tranquilli, W.J. & Benson, G.J., pp.448–478. Williams and Wilkins, Baltimore, MD.
16. Natalini, C.C. & Robinson, E.P. (2000) Evaluation of the analgesic effects of epidurally administered morphine, alfentanil, butorphanol, tramadol, and U50488H in horses. *American Journal of Veterinary Research*, 31, 1579–1586.
17. Natalini, C.C. & Robinson, E.P. (2003) Effects of epidural opioid analgesics on heart rate, arterial blood pressure, respiratory rate, body temperature, and behaviour in horses. *Veterinary Therapeutics : Research in Applied Veterinary Medicine*, 4, 364–375.
18. Natalini, C.C. & Linardi, R.L. (2006) Analgesic effects of epidural administration of hydromorphone in horses. *American Journal of Veterinary Research*, 67, 11–15.
19. Natalini, C.C., Polydoro, A.S. & Linardi, R.L. (2006) Analgesic effects of subarachnoidally administered hyperbaric opioids in horses. *American Journal of Veterinary Research*, 67, 941–946.
20. Gibaldi M. & Prescott L. (1983) *Handbook of Clinical Pharmacokinetics*. Australia: ADIS Health Science Press, Australia, p.1208.
21. Yamaoka, K., Nakagawa, T. & Uno, T. (1978) Statistical moments in pharmacokinetics 1. *Journal of Pharmacokinetics and Biopharmaceutics*, 6, 547–558.
22. Birkett, D.J. (2002) *Pharmacokinetics made easy – Revised ed.* McGraw-Hill Australia Pty Limited, North Ryde, 132 p.
23. Gourlay G.K, Wilson, P.R. & Glynn, C.J. (1982) Pharmacodynamics and pharmacokinetics of methadone during the perioperative period. *Anesthesiology*, 57, 458–467.
24. Gourlay, G.K., Willis, R.J. & Wilson, P.R. (1984) Postoperative pain control with methadone: Influence of supplementary methadone doses and blood concentration-response relationships. *Anesthesiology*, 61, 19–26.
25. Gourlay, G.K, Willis, R.J. & Lamberty, J. (1986) A double-blind comparison of the efficacy of methadone and morphine in postoperative pain control. *Anesthesiology*, 64, 322–327.
26. Walker, A.F. (2007) Sublingual administration of buprenorphine for long-term analgesia in the horse (Short Communication). *The Veterinary Record*, 160, 808–809.
27. Shargel, L., Wu-Pong, S. & Yu, A.B.C. (2005) *Applied Biopharmaceutics & Pharmacokinetics*, 5th edn. Mc Graw-Hill, Medical Publishing Division, White Plains p.768.

**CHAPTER 3.**  
**BIOAVAILABILITY AND PHARMACOKINETICS OF ORAL AND INJECTABLE**  
**FORMULATIONS OF METHADONE AFTER INTRAVENOUS, ORAL, AND**  
**INTRAGASTRIC ADMINISTRATION IN HORSES**

### 3.1 Introduction

Opioids are in general, effective analgesic drugs used to treat acute and chronic pain in humans and animals. However; opioids have limited use in equine medicine due to severe undesirable side effects, especially after intravenous administration. Therefore, alternative routes for opioid administration may be a way to achieve satisfactory therapeutic effects with minimal adverse side effects in horses<sup>1-4</sup>. Oral administration is probably the easiest and most convenient and cost-effective route for drug delivery and it may be associated with less opioid-induced side effects. However, the oral disposition including absorption of opioid drugs is variable in horses due to factors described to affect drug bioavailability, such as drug properties, gastrointestinal and drug pH, gastrointestinal physiology and genetic factors like transporter proteins<sup>5</sup>.

Methadone is a very effective opioid agent which has unique properties to treat severe acute and chronic, neuropathic, and cancer-related pain in humans<sup>6-9</sup>. An inter- and intra-individual variability in the disposition of methadone has been described after oral administration in humans. Although methadone has physicochemical characteristics favorable for good absorption, its oral bioavailability has been reported to be between 30 and 80%<sup>10</sup>. Methadone pharmacokinetics (PK) are characterized by rapid absorption, wide tissue distribution, and long elimination half-life in people<sup>11,12</sup>. Methadone is extensively metabolized in human hepatocytes and enterocytes (N-demethylation) by the cytochrome CYP-450 enzymes, primarily CYP3A4 and to a lesser extent CYP2D6 to inactive metabolites<sup>6,8,13-15</sup>. In addition, the CYP2B has been reported to be the primary metabolizing enzyme in humans<sup>16</sup>. However in dogs, methadone has poor oral bioavailability, short elimination half-life, and rapid clearance, and specific metabolic enzymes and metabolites are still not fully characterized<sup>17,18</sup>.

As a synthetic  $\mu$ -opioid receptor agonist and an N-methyl D-aspartate (NMDA) receptor antagonist, methadone is usually used as a racemic mixture of levo (*l*)- and dextro (*d*)-isomers, and it was recently investigated in horses after single oral administration. Concentrations of methadone above the effective or therapeutic concentration reported for humans (33 to 59 ng/mL) were measured in the serum of horses and no side effects were observed. Oral PK of methadone was characterized in horses by short elimination half-life, rapid clearance, and small volume of distribution<sup>19</sup>.

Methadone is a highly lipophilic drug comprising physicochemical characteristics related to high solubility and permeability; these characteristics favor oral or gastrointestinal drug absorption and therefore, oral administration of the drug<sup>20,21</sup>. In horses, oral administration of methadone could potentially benefit equine pain management by limiting typical opioid-induced side effects such as excitation and stasis of gastrointestinal motility usually experienced after intravenous administration. However, drug absorption and oral disposition of methadone are still not completely described in horses. The purposes of this study were to characterize the pharmacokinetics of methadone after intravenous, oral, and intragastric administration and to determine drug bioavailability in order to understand the absorption of methadone in horses.

## **3.2 Material and Methods**

### **3.2.1 Animals**

Six healthy adult horses (3 gelding and 2 female Thoroughbreds and 1 gelding Quarter horse) with mean ( $\pm$ SD) body weight of 504.6 kg ( $\pm$ 39.37) and mean ( $\pm$ SD) age of 5.5 years ( $\pm$ 1.87) were evaluated. As a selection criterion, horses did not receive any medication for at least four weeks prior to the study. Horses were placed in stalls for acclimation two days before each study and had free access to pasture during the washout period. Body weight was

determined before each crossover for drug calculation. Horses received a complete pelleted ration twice daily and were fasted for 12 hours prior to drug administration. They were again fed six hours after drug administration and had free access to water during the entire study. A 14-gauge catheter (B.D. Angiocath IV catheter, 14-gauge x 5.25 inch, Becton Dickinson Infusion Therapy System Inc., Franklin Lakes, NJ, USA) was placed in the left jugular vein for blood collection. This study was approved by the Louisiana State University Institutional Animal Care and Use Committee.

### 3.2.2 Study Design

In a randomized crossover design, horses received a single dose (0.15 mg/kg) of the oral or injectable formulation of methadone hydrochloride (Methadone HCl Oral Solution<sup>®</sup>, USP 10 mL per 5 mL, Roxane Laboratories, Columbus, OH, USA; Methadone HCl Injection, USP 10 mg/mL, AAIPharma, Wilmington, NC, USA respectively) by the oral route (directly into the mouth) or intragastric administration via nasogastric tube (NG). For intravenous administration, only the injectable formulation was administered via venipuncture in the opposite vein from sampling (Table 3.1). A two-week washout period was used between each treatment. Blood samples (10 mL) were collected into blood collection tubes (BD Vacutainer<sup>®</sup> Blood Collection Tube, no additive, BD, Franklin Lakes, NJ, USA), and remained at room temperature for 60 minutes. After centrifugation at 2808 g for 10 minutes, serum was transferred to sterile propylene tubes (Biomed Resource Inc., Torrance, CA, USA) and stored at -20°C until analysis. Horses were clinically monitored during the study for possible side effects. Cardiac and respiratory rates (auscultation and respiratory movements count) were recorded and behavior, excitation or sedation (low head position), and locomotor activity were observed. All horses were returned to the herd after conclusion of the study.

### 3.2.3 Serum Sampling and Clinical Evaluation

Blood was collected from the jugular vein catheter at 0, 15, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360 minutes after drug administration. Clinical evaluation was performed at the same time points 0, 15, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360 minutes and at 9, 12, and 24 hours after the completion of the study.

**Table 3.1 – Formulations and Routes of Methadone Administration in Horses**

Route of Administration	Methadone Formulation	
	Oral	Injectable (iv)
Intravenous (IV)	Not applied	IV
Oral	oral-ORAL	iv-ORAL
Intragastric (NG)	oral-NG	iv-NG

### 3.2.4 Serum Analysis

The concentration of methadone in equine serum samples was determined using Gas Chromatography/Mass Spectrometry (GC/MS) analysis (Agilent Technologies, Wilmington, DE; 6890 Series GC System with a 5973 MS Detector) as previously described<sup>14</sup>. Briefly, serum samples were aliquotted into separate tubes, along with a method blank (water) and serum blanks with and without internal standard, the DL-Methadone-*d*<sub>3</sub> (Isotec Inc., Miamisburg, OH, USA). Methadone working standard (internal standard in methanol) was prepared in concentrations of 0.1, 1 and 10 µg/mL and calibration standards (working standard in drug free equine serum) at concentrations of 2, 5, 10, 20, 50, 100, 250 and 500 ng/mL. The internal standard was added to aliquots of each control and test sample. After extraction and centrifugation, samples were evaporated under a continuous stream of dry nitrogen (N-EVAP, Organization Associates, South Berlin, MA, USA) and the resulting residue dissolved in methylene chloride and transferred to a micro-injection vial for GC/MS analysis. Concentrations were determined by producing a

calibration curve using the peak area ratios of the analyte (methadone, 294  $m/z$ ) to the internal standard (methadone- $d_3$ , 297  $m/z$ ). Chromatographic data were processed using Agilent software (Agilent ChemStation, Foster, CA, USA). Limit of quantification (LOQ) was defined as the lowest concentration within approximately 20% of precision (LOQ: 2 ng/mL). Inter- and intra-assay coefficients of variation were 3.30–3.50% and 1.50–1.55%, respectively. The minimal acceptable correlation ( $R^2$ ) for standard curves was 0.998 with  $R^2$  mean (SD) of 0.999 (0.001). Analysis was performed by the Analytical Systems Laboratory, School of Veterinary Medicine, Louisiana State University.

### 3.2.5 Pharmacokinetics Analysis

A non-compartmental model was fitted to the serum concentration data of each horse to generate individual time-serum concentration curves. The linear trapezoidal model with linear interpolation was used to estimate non-compartmental PK parameters such as terminal or elimination half-life ( $t_{1/2}$ ), area under the concentration-time curve from 0 to the last time point ( $AUC_{0-t}$ ), and mean residence time ( $MRT_{0-INF}$ )<sup>22,23</sup>. Systemic or total body clearance ( $CL/F$ ) and apparent volume of distribution ( $V_d/F$ ) were corrected for bioavailability for oral and NG routes<sup>22,24</sup>. A linear regression analysis estimated the first order rate constant associated with the terminal (log-linear) portion of the curve ( $\lambda_z$ ) using up to 13 serum log concentration-time points. Estimated maximum serum drug concentration ( $C_{max}$ ) and time to maximum concentration ( $T_{max}$ ) were determined directly from the estimated concentration-time curves obtained for different doses. Absolute bioavailability ( $F$ ) was calculated for each treatment (oral-ORAL, oral-NG, iv-ORAL, iv-NG) as the ratio of total AUC from each formulation-route combination to the total AUC from the IV administration and expressed as  $F^{24}$ :

$$F_{Treatm} = AUC_{Treatm} / AUC_{IV}$$



where,  $AUC_{Treatm}$  is the area under the curve for each treatment (oral-Oral, oral-NG, iv-Oral, iv-NG), AUC is the area under the curve for IV administration.

Analysis was performed using the WinNonlin computer software Version 5.1 (Pharsight Corporation, Mountain View, CA).

### 3.2.6 Statistical Analysis

Statistical analysis was performed using SAS 9.1 (SAS Institute, Cary, NC.). Parameters were log transformed to follow normal distribution and statistical differences between the PK parameters ( $AUC_{0-t}$ ,  $MRT_{0-INF}$ ,  $t_{1/2}$ ,  $CL/F$ ,  $V_d/F$ ,  $C_{max}$ ,  $T_{max}$ , and  $F$ ) were analyzed using one-way analysis of variance (ANOVA), repeated measures and randomized block designs with horse as a blocking factor. Tukey's test was used as the post hoc test for mean comparison of the estimated PK parameters between treatments with significance set at  $P < 0.05$ . The interaction of horse with the given effect combination was used as the error term for testing those effects in the model.

### 3.3 Results

Methadone was well tolerated by all horses after oral, intragastric, or IV administration. No behavioral changes or opioid-induced side effects such as excitement, sedation, increased locomotor activity, and decrease of gastrointestinal motility were observed during the 24-hour study period. Physiological parameters for cardiac and respiratory rates were in the normal range during the entire period of study.

Methadone was first measured in the serum of all horses at 15 minutes and drug was detected above the LOQ (2ng/mL) during six hours after administration by all routes. Serum concentration vs. time curves generated after oral and intragastric administration were characterized by a biphasic profile with rapid absorption and elimination phases describing a first order process. Considering complete absorption (100%), the curve for IV administration showed

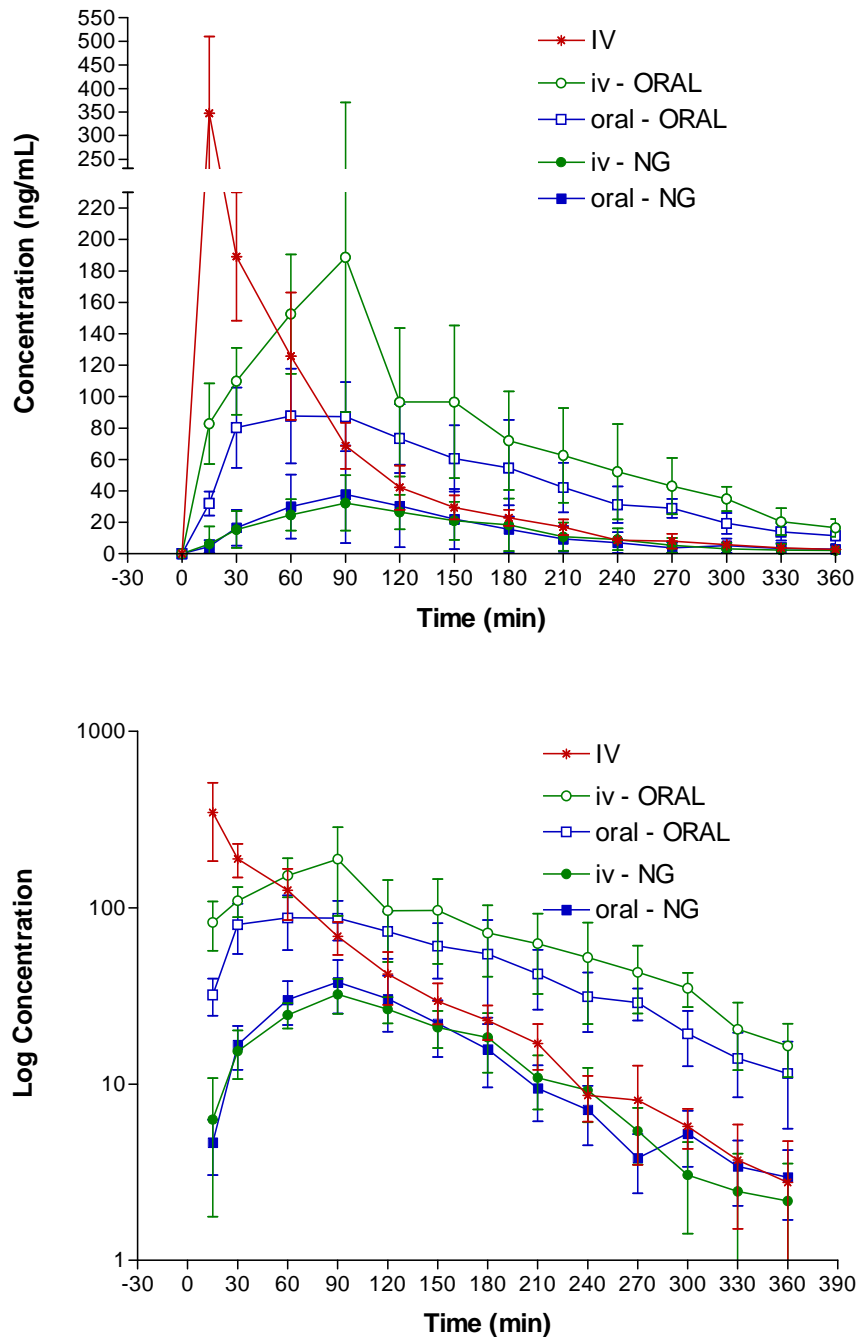
rapid distribution and elimination of the drug. The area under the serum concentration time curve for IV administration of methadone ( $AUC_{iv-IV}$ ) was used to estimate drug bioavailability for both oral and NG routes (Figure 3.1).

Values (mean  $\pm$ SD) estimated PK parameters after IV, oral, and intragastric administration were determined (Table 3.2). Estimated elimination half-life,  $MRT_{0-INF}$ , and  $T_{max}$  did not differ among solutions or among routes of administration. However,  $AUC_{0-t}$ ,  $CL/F$ ,  $V_d/F$ ,  $C_{max}$ , and  $F$  were significantly different across treatments. The  $AUC$  and  $F$  were also significantly different between oral and injectable formulations after oral administration but not by NG route ( $P < 0.05$ ). The estimated  $T_{max}$  ( $\pm$ SD) for all administrations was between  $65.5 \pm 50.93$  min and  $105.0 \pm 41.35$  min. Methadone oral bioavailability was approximately three times higher than that of the intragastric  $F$ .

### 3.4 Discussion

The single dose of 0.15 mg/kg was selected based on a previous study carried out in our laboratory that demonstrated the pharmacokinetics of methadone are dose-independent and oral administration was not associated with opioid-induced side effects<sup>19</sup>. Effective or therapeutic concentrations for methadone have not been reported in horses; however, a range of 33 to 59 ng/mL has been established as the correlation of plasma concentration and methadone efficacy in people<sup>10,25,26</sup>. As well as in the previous study, serum concentrations of methadone after oral and intragastric administration in this investigation were equivalent or higher to the effective concentration range reported for people.

In this present study, methadone was well tolerated by horses with no adverse or induced side effects observed after IV, oral or NG route, including excitement, respiratory depression, increased locomotor activity, or decreased gastrointestinal motility.



**Figure 3.1 – Serum Concentration vs. Time Curves for Intravenous, Oral, and Intragastic Administration of Methadone in Horses**

Mean ( $\pm$ SD) of the estimated serum concentrations of methadone (ng/mL) of five horses over 6 hours after single administration (0.15 mg/mL) of the injectable (iv) and oral (oral) formulations by intravenous (IV), oral (ORAL), and nasogastric (NG) routes. Methadone concentration is in ng/mL (a) and using natural log (b). Points are connected by a line for better visualization.

**Table 3.2 – Pharmacokinetic Parameters of Intravenous, Oral, and Intragastric Administration of Methadone**

	Pharmacokinetic Parameters							
	AUC <sub>0-t</sub> (ng.min/mL)	MRT <sub>0-INF</sub> (min)	t <sub>1/2</sub> (min)	CL (mL/min/kg)	V <sub>d</sub> (L/kg)	C <sub>max</sub> (ng/mL)	T <sub>max</sub> (min)	F
<b>IV</b>	18791 (2726.2)	78.8 (15.69)	58.3 (8.71)	8.0 (0.0012)	0.7 (0.13)	NA	NA	NA
	AUC <sub>0-t</sub> (ng.min/mL)	MRT <sub>0-INF</sub> (min)	t <sub>1/2</sub> (min)	CL/F (mL/min/kg)	V <sub>d</sub> /F (L/kg)	C <sub>max</sub> (ng/mL)	T <sub>max</sub> (min)	F
<b>iv-Oral</b>	28469 (9249.5) a	150.2 (6.62) a	72.5 (13.47) a	5.0 (0.0016) a	0.6 (0.19) a	195.0 (93.89) a	80.0 (15.49) a	1.5 (0.57) b
<b>oral-Oral</b>	17659 (4680.6) b	162.8 (23.99) a	78.1 (30.08) a	8.0 (0.003) a	0.9 (0.3) a	100.8 (20.66) a	65.50 (50.93) a	0.96 (0.29) a
<b>iv-NG</b>	5068 (2501.1) c	149.2 (32.59) a	62.4 (33.93) a	35.0 (0.02) b	2.8 (1.35) b	35.1 (17.31) b	105.0 (41.35) a	0.3 (0.14) c
<b>oral-NG</b>	5449 (4048.1) c	154.7 (49.09) a	65.9 (31.46) a	40.0 (0.03) b	3.2 (1.73) b	41.58 (31.95) b	80.0 (30.98) a	0.3 (0.22) c

The table comprises mean (SD) of the estimated non-compartmental pharmacokinetic parameters obtained after a single dose (0.15 mg/kg) of the injectable (iv) and oral (oral) formulations of methadone HCl administered intravenously (IV), orally (ORAL), and intragastric tube (NG) to six horses. Statistical differences between treatments are indicated by different letters ( $P < 0.05$ ). AUC<sub>0-t</sub> – Area under the serum concentration-time curve from 0 to the last time measured, MRT<sub>0-INF</sub> – Mean residence time extrapolated to the infinity, t<sub>1/2</sub> – elimination half-life, CL/F – total body clearance corrected for bioavailability, V<sub>d</sub> /F – apparent volume of distribution corrected for bioavailability, C<sub>max</sub> – maximum drug concentration, T<sub>max</sub> – time to maximum concentration, F - bioavailability.

As described for other species, individual variability regarding the pharmacokinetics of methadone was also observed in horses<sup>6,8,14,17,18</sup>. Methadone had a short  $t_{1/2}$  of approximately 1 hour, short MRT, rapid CL/F, and large  $V_d/F$  in horses. Studies in beagles and greyhound dogs showed similar parameters including low oral bioavailability after single IV or oral administration of methadone. But in contrast to our study, dogs showed minimal side effects and plasma concentration below the therapeutic concentration (<40 ng/mL) two hours after drug administration<sup>17,18</sup>.

To our knowledge, this is the first study that investigates oral and intragastric administration of methadone in horses. We intended to determine drug bioavailability and possibly predict the absorption of methadone in horses. Highly lipophilic compounds like methadone are expected to be absorbed via passive transcellular transport mainly from the small intestine after oral (and intragastric) administration due to its large surface area and pH between 6 and 7.5<sup>5,27,28</sup>. Besides physiological properties of membranes, biochemical drug properties including lipid solubility, degree of ionization, acid dissociation constant pka, solution pH and formulation, and size and molecular weight of the compound are determinant factors for drug absorption<sup>27</sup>. Methadone comprises characteristics related to high solubility and permeability such as low molecular weight (below 500 Da), no hydrogen-bond donors, single oxygen and nitrogen molecules, and octanol/water partition coefficient (log P) less than 5, which likely favors drug absorption. However, this study suggested the absorption of methadone through the intestinal mucosa was limited. Low AUC and F were observed after intragastric administration of both oral and injectable formulations and it could be due to the first-pass metabolic effect or other limiting factor for drug absorption. Genetic factors including protein transporters can play a major role on absorption and disposition of drugs and the expression of P-glycoprotein (P-gp) in

the apical membrane of enterocytes may be directly related to it. As an energy (ATP)-dependent efflux transmembrane protein transporter, P-gp is described to limit absorption of several drugs by transporting them out of cells and back to the intestinal lumen. This protein is constitutively expressed in diverse tissues and species and it may interfere with the disposition of several drugs including opioids<sup>29-35</sup>. However, little is known about this protein and its role in the absorption and disposition of methadone in horses.

Compared to the NG route, methadone administered orally showed higher plasma concentrations, AUC, and F; therefore, the oral cavity appeared to contribute considerably to the absorption of methadone through the oral mucosa. Methadone is a weak base with pKa of approximately 9.2 and the pH of 7-8 measured in the oral cavity (saliva) of horses probably favored drug transport across the oral mucosa. Environmental pH and drug pka determine the degree of ionization of the drug and the higher unionized fraction of the drug, the greater its liposolubility and permeability/absorption. In addition, the high venous blood flow under the tongue probably an important factor to favor a rapid and more complete absorption of methadone from the oral cavity<sup>27,36</sup>.

One of this study's most interesting findings was a prolonged drug serum concentration vs. time curves after oral administration and oral F for the injectable formulation of methadone greater than 1.0. This observation could be due to a superimposition of oral and intestinal absorption when part of the drug is swallowed and reaches the small intestine. However, oral administration could potentially result in higher serum concentrations than the systemic concentration with sampling from the jugular vein as the method used in this study. Since the jugular vein provides venous drainage for the head, the parameters could be overestimated.

Methadone HCl has a molecular weight of 345.19 g/mol and both solutions used in this study differ in their composition only regarding the inactive ingredients which apparently do not interfere with the disposition of the drug. However, the injectable formulation (pH 3.28) of methadone seemed to be better absorbed compared to the oral formulation (pH 2.62) by oral route possibly due a higher dissociation of unionized fraction favored by the higher delivery solution's pH. In addition, horses salivated more with the administration of the oral formulation by mouth and this could explain the different serum concentrations and F between formulations. We believe the flavoring component of the inactive ingredients confers a strong smell and taste to the solution which was not well accepted or appreciated by the horses. Possibly, it could have caused the solution to be swallowed faster having shorter time in contact with the oral mucosa or some of the drug to be lost in the saliva.

### 3.5 References

1. Skarda, R. T. (1996) Local and regional anesthetic and analgesic techniques: horses. In: Thurmon JC, Tranquilli WJ, Benson GJ, eds. *Lumb & Jones' veterinary anesthesia*. Baltimore, MD: Williams and Wilkins. p.448-478.
2. Natalini, C. C., Robinson, E. P. (2000) Evaluation of the analgesic effects of epidurally administered morphine, alfentanil, butorphanol, tramadol, and U50488H in horses. *American Journal of Veterinary Research*. 61(12), 1579-1586.
3. Natalini, C. C., Robinson, E. P. (2003) Effects of epidural opioid analgesics on heart rate, arterial blood pressure, respiratory rate, body temperature, and behavior in horses. *Vet.Therapeutics*. 4(4), 364-375.
4. Natalini, C. C., Polydoro, A. S., Linardi, R. L. (2006) Analgesic effects of subarachnoidally administered hyperbaric opioids in horses. *American Journal of Veterinary Research*. 67(6), 941-946.
5. Davis, J.L., Little, D., Blikslager, A.T., Papich, M.G. (2006) Mucosal permeability of water-soluble drugs in the equine jejunum: a preliminary investigation. *J. vet. Pharmacol. Therap*, 29, 379-385.
6. Eap, C.B., Buclin, T., Baumann, P. (2002) Interindividual Variability of the Clinical Pharmacokinetics of Methadone. *Clinical Pharmacokinetics*, 41(14), 1153-1193.

7. Inturrisi, C.E. (2005) Pharmacology of methadone and its isomers. *Minerva Anesthesiology*, 71, 435–437.
8. Lugo, R.A., Satterfield, K.L. & Kern, S.E. (2005) Pharmacokinetics of methadone. *Journal of Pain & Palliative Care Pharmacotherapy*, 19, 13–24.
9. Kharasch, E.D., Hoffer, C., Whittington, D., Walker A., Bedynek, P.S. (2009) Methadone pharmacokinetics are independent of cytochrome P4503A (CYP3A) activity and gastrointestinal drug transport. *Anesthesiology*, 110, 660-672.
10. Gourlay, G.K., Cherry, D.A., Cousins, M.J. (1986). A comparative study of the efficacy and pharmacokinetics of oral methadone and morphine in the treatment of severe pain in patients with cancer. *Pain*, 25(3), 297-312.
11. Dale O, Hoffer C, Sheffels P et al. (2002) Disposition of nasal, intravenous, and oral methadone in healthy volunteers. *Clin Pharmacol Ther*, 72, 536–545.
12. Dale, O., Sheffels, P., Kharasch, E.D. (2004) Bioavailabilities of rectal and oral methadone in healthy subjects. *Br J Clin Pharmacol*, 58 (2), 156-162.
13. Foster, D.J., Somogyi, A.A. & Bochner, F. (1999) Methadone N-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4. *British Journal of Clinical Pharmacology*, 47, 403–412.
14. Boulton, D.W., Arnaud, P. & DeVane, C.L. (2001) Pharmacokinetics and pharmacodynamics of methadone enantiomers after a single oral dose of racemate. *Clinical Pharmacology and Therapeutics*, 70, 48–57.
15. Oda, Y. & Kharasch, E.D. (2001) Metabolism of methadone and levoalpha-acethylmetadol (LAAM) by human intestinal cytochrome P450 3A4 (CYP3A4), potential contribution of intestinal metabolism to presystemic clearance and bioactivation. *The Journal of Pharmacology and Experimental Therapeutics*, 298, 1021–1032.
16. Totah, R. A., Sheffels, P., Roberts, T., Whittington, D., Thummel, K., Kharasch, E. D. (2008) Role of CYP2B6 in stereoselective human methadone metabolism. *Anesthesiology*. 108(3), 363-374.
17. Kukanich, B., Lascelles, B.D., Aman, A.M., Mealey, K.L. & Papich, M.G. (2005) The effects of inhibiting cytochrome P450 3A, p-glycoprotein, and gastric acid secretion on the oral bioavailability of methadone in dogs. *Journal of Veterinary Pharmacology and Therapeutics*, 28, 461–466.
18. Kukanich, B., Borum, S.L. (2008) The disposition and behavioral effects of methadone in Greyhounds. *Veterinary Anaesthesia and Analgesia*, 35, 242-248.



19. Linardi, R.L., Stokes, A.M., Barker, S.A., Short, C., Hosgood, G., Natalini, C.C. (2009). Pharmacokinetics of the injectable formulation of methadone hydrochloride administered orally in horses. *J. vet. Pharmacol. Therap*, 32, 492-497.
20. Curatolo, W. (1998) Physical chemical properties of oral drug candidates in the discovery and exploratory development settings. *PSTT*. 1(9), 387-393.
21. Davis, J. L., Papich, M. G., Weingarten, A. (2006) The pharmacokinetics of orbifloxacin in the horse following oral and intravenous administration. *J. vet. Pharmacol. Therap*, 29, 191-197.
22. Gibaldi M., Prescott L. (1983) *Handbook of Clinical Pharmacokinetics*. Australia: ADIS Health Science Press, Australia, p.1208.
23. Yamaoka, K., Nakagawa, T., Uno, T. (1978) Statistical moments in pharmacokinetics 1. *Journal of Pharmacokinetics and Biopharmaceutics*, 6, 547–558.
24. Birkett, D.J. (2002) *Pharmacokinetics made easy – Revised ed.* McGraw-Hill Australia Pty Limited, North Ryde, 132 p.
25. Gourlay, G.K., Wilson, P.R., Glynn, C.J. (1982) Pharmacodynamics and pharmacokinetics of methadone during the perioperative period. *Anesthesiology*, **57**(6), 458-467.
26. Gourlay, G.K., Willis, R.J. & Wilson, P.R. (1984) Postoperative pain control with methadone: Influence of supplementary methadone doses and blood concentration–response relationships. *Anesthesiology*, 61, 19–26.
27. Batheja, P., Thakur, R., Michniak, B. (2007) Basic biopharmaceutics of buccal and sublingual absorptions, chapter 9, 175-202. In: Touitou, E., Barry, B.W. Enhancement in drug delivery. CRC Press, Taylor & Francis Group, LLC, USA. p.615.
28. Wells, S. M., Glerum, L. E., Papich, M. G. (2008) Pharmacokinetics of butorphanol in cats after intramuscular and buccal transmucosal administration. *American Journal of Veterinary Research*. 69(12), 1548-1554.
29. Thompson, S.J., Koszdin, K., Bernards, C.B. (2000) Opioid-induced analgesia is increased and prolonged in mice lacking P-glycoprotein. *Anesthesiology*. 92(5), 1392-1399.
30. Lotsch, J., Sharke, C., Tegeder, I., Geisslinger, G. (2002) Drug Interactions with patient-controlled analgesia. *Clinical Pharmacokinetics*. 41(1), 31-57.
31. Katsura, T., Inui, K-i. (2003) Intestinal absorption of drugs mediated by drug transporters: Mechanisms and Regulation. *Drug Metab. Pharmacokin*, 18(1), 1-15.

32. Sun, J., He, Z.G., Chen, G., Wang, S.J., Hao X.H., Zou M.J. (2004) Multidrug resistance P-glycoprotein: crucial significance in drug disposition and interaction. *Medical Science Monitor*. 10(1), RA5-14.
33. Wang, J.S., Ruan, Y., Taylor, R.M., Donovan, J.L., Markowitz J.S, DeVane, C.L. (2004) Brain penetration of methadone (R)-and(S)-enantiomers is greatly increased by P-gp deficiency in the blood-brain barrier of Abcb1a gene knockout mice. *Psychopharmacologia*, 173(1), 132-8.
34. Fakhoury, M., Litalien, C., Medard, Y., Cavé, H., Ezzahir, N., Peuchmaur, M., Jacqz-Aigrain E. (2005) Localization and mrna expression of cyp3a and P-glycoprotein in human duodenum as a function of age. *Drug Metab. Dispos.*, 33, 1603-1607.
35. Zimmermann, C., Gutmann, H., Hruz, P., Gutzwiller, JP., Beglinger, C., Drewe, J. (2005) Mapping of multidrug resistance gene 1 and multidrug resistance associated protein isoform 1 to 5 mRNA expression along the human intestinal tract. *The American Society for Pharmacology and Experimental Therapeutics*. 33(2), 219-224.
36. Hardman, J.G. & Limbird, L.E. (2001) Goodman & Gilman's The Pharmacological Basis of Therapeutics. United States: McGraw-Hill, US, p. 2148.
37. Weinberg, D.S., Intrussi, C.E., Reidenberg, B., Moulin, D.E., Nip, T.J., Wallenstein, S., Houde, R.W., Foley, K.M. (1988) Sublingual absorption of selected opioid analgesics. *Clinical pharmacology and therapeutics*, 44(3), 335-342.

**CHAPTER 4.**  
**IDENTIFICATION AND SEQUENCING OF EQUINE MULTIDRUG RESISTANCE**  
**(MDR1) GENE AND DIFFERENTIAL mRNA AND P-GLYCOPROTEIN (P-gp)**  
**EXPRESSION ACROSS TISSUES IN HORSES**

#### 4.1 Introduction

P-glycoprotein (P-gp) is an important membrane transporter protein encoded by the multi-drug resistance (MDR1) gene which belongs to the subfamily B of the ATP-Binding Cassette (ABC) superfamily of membrane transporter proteins. This protein is usually over-expressed in tumor cells and plays a major role in cell resistance to chemotherapeutic drugs leading to treatment failure in humans. The P-gp is constitutively expressed in non-tumor cells of intestine, central nervous system, kidney and other tissues<sup>1-14</sup>. As an energy (ATP)-dependent efflux pump protein, P-gp limits entry of xenobiotics or other compounds into circulation and/or increases elimination by transporting them out of cells, playing a role in the detoxification<sup>1-8,15-17</sup>. Furthermore, MDR1 gene and P-gp also play a critical role in the absorption and disposition of pharmaceutical agents which have a significant impact on therapeutic responses<sup>5,7,16,18-21</sup>.

Expressed in blood-tissue barriers, P-gp limits entry and accumulation of compounds in tissues within the central nervous system, fetus, and the testis<sup>16,19,20,22-24</sup>. Also, this transporter protein is able to enhance drug elimination through bile and urine, as a result of its expression in the canalicular membrane of hepatocytes and luminal membrane of proximal tubule cells in the kidneys, respectively<sup>14,16</sup>. In the small intestine, expression of P-gp usually results in decreased drug absorption after oral administration; however, P-gp can modulate intestinal drug metabolism by increasing the exposure of drugs to intracellular enzyme Cytochrome P3A4 (CYP3A4) due to their common affinity to some substrates<sup>4,8,15,24,26</sup>. The expression of P-gp is directly associated with low plasma/tissue drug concentration and compromised therapeutic efficacy due to the interference on the pharmacokinetics of the majority of opioids<sup>26-30</sup>.

Gene or protein deficiency, mutation of MDR1 gene, and altered expression and/or protein function have also important clinical impact and are more associated with high drug

plasma concentration and drug intoxication as first clinically demonstrated in Collie dogs<sup>31,32</sup>. The influence of MDR1 and P-gp as well as their absence on drug pharmacokinetics and pharmacodynamics have been supported by *in vitro* and *in vivo* studies in many species including humans, dogs, rabbits, and mice<sup>3,6,32-37</sup>, but not in horses. There was no sequence available for the equine MDR1 gene in the Nucleotide Sequence Database (GenBank) of the National Center for Biotechnology Information (NCBI).

An *in vivo* investigation (unpublished data) recently suggested the intestinal absorption of opioid is limited in horses after oral drug administration and we hypothesize that it could be due to high levels of MDR1-P-gp expression in the equine small intestine. However, the expression and activity of MDR1 gene and P-gp in horse tissues was unknown. Therefore, the purpose of this study was to identify and sequence the equine MDR1 gene and to determine the differential gene and P-gp expression and distribution across tissues in horses.

## **4.2 Materials and Methods**

### **4.2.1 PART 1. Identification and Sequencing of the Equine MDR1 Gene**

#### **4.2.1.1 Design of Primers**

Forward and reverse gene-specific primers were randomly designed using Mac Vector 65.3all software (Applied Biosystems, Foster, CA, USA) from three different regions of the known rabbit MDR1 gene sequence (*Oryctolagus cuniculus* multi-drug resistance P-glycoprotein 1 [ABCB1] mRNA, complete cds. GenBank:AY360144.3) available in the Nucleotide Sequence Database (GenBank) of the National Center for Biotechnology Information (NCBI) (Table 4.1). Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) and used at a final concentration of 20 uM.

**Table 4.1 – Design of Forward and Reverse Primers (GenBank AY360144)**

<b>Gene</b>	<b>Accession Number (GenBank)</b>	<b>Position</b>	<b>Primer 5' – 3'</b>	<b>Sequence</b>
ABCB1	AY360144	1027–1050	Forward 1	TCTCTGTGGGTGTTGCTTTCCTGC
		1594–1574	Reverse 1	TCAGCGATTGTGGTGGCGAAC
		3115–3137	Forward 2	GCTCATTTGCCCCTGA CTATGCC
		3903–3879	Reverse 2	CGCACTCTTTGACTCTGCCGTTTTG
		3385–3407	Forward 3	TCATTGAGCGGTTCTACGACCCC
		4015–3991	Reverse 3	AACAAGTATCTCCCATCTCCCACGG

Gene available in the Nucleotide Sequence Database (GenBank) of the National Center for Biotechnology Information (NCBI).

Table shows the three random sequences of forward and reverse primers designed using the known rabbit MDR1 gene sequence (*Oryctolagus cuniculus* multi-drug resistance P-glycoprotein 1 [ABCB1] mRNA, complete sequence cds. GenBank: AY360144.3).

#### 4.2.1.2 Template

##### 4.2.1.2.1 Tissue Collection

Samples of the ileum from an adult horse with no evidence of gastrointestinal disease were collected 30 minutes after humane euthanasia. Tissues (0.25 mg) were immediately stored in polyethylene micro tubes containing 0.75 mL of TRI REAGENT<sup>®</sup> LS – RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Cincinnati, OH, USA) to prevent RNA degradation. Tubes were stored at -80°C until analysis. This study was approved by the Louisiana State University Institutional Animal Care and Use Committee.

##### 4.2.1.2.2 RNA Isolation

Total RNA from ileum tissue was isolated using TRI REAGENT<sup>®</sup> LS as per the manufacturer's protocol. Briefly, after tissue homogenization, RNA (colorless upper aqueous phase) was separated from DNA and proteins (interphase and lower organic phase – red phenol-chloroform) using chloroform, and precipitated by isopropanol. RNA samples were treated with DNase<sup>®</sup> (TURBO<sup>™</sup> DNase, Ambion, Austin, TX, USA) to remove possible genomic DNA

contamination. RNA concentration and purity were determined by spectrophotometry using optical density at 260 and 280 nm, OD<sub>260</sub> and OD<sub>280</sub> respectively.

#### 4.2.1.2.3 cDNA Synthesis

First-strand complementary DNA (cDNA) was synthesized from total RNA using the Ready-To-Go You-Prime First-Strand Beads kit<sup>®</sup> (GE Healthcare, Pittsburg, PA, USA) as per the manufacturer's instructions. Complementary DNA was used as a template for the Polymerase Chain Reaction.

#### 4.2.1.3 Polymerase Chain Reaction (PCR)

PCR was performed under ideal conditions (denaturation: 94°C – 30 seconds, annealing: 62°C – 30 seconds, and extension: 72°C – 1 minute, 33 cycles) using the GeneAmp PCR System 9600<sup>®</sup> (Perkin Elmer, Waltham, MA, USA). For PCR reaction, cDNA (template) was mixed with forward and reverse primers, deoxynucleoside triphosphate (dNTP), buffer solution, Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), and sterile water to bring the solution to a volume of 50 µL. The reaction was carried out in duplicates in a 96 well plate. The specificity of amplification was determined by agarose electrophoresis to confirm product size and to detect the presence of non-specific amplification products.

#### 4.2.1.4 Plasmid pcDNA 3.1/MDR1

MDR1 was cloned into pcDNA3.1 plasmid vector (pcDNA3.1/V5-His-<sup>®</sup>TOPO<sup>®</sup> TA Expression Kit, Invitrogen, Carlsbad, CA, USA) in order to be sequenced. DNA fragments (PCR product) were recovered from agarose gel using Zymoclean<sup>™</sup> Gel DNA Recovery Kit (Zymo Research Corp, Orange, CA, USA) as per the manufacturer's instructions. The DNA (MDR1) was then cloned into pcDNA3.1 plasmid vector and chemically transformed into *E. coli* competent cells (One Shot<sup>®</sup> TOP10, Invitrogen, Carlsbad, CA, USA). After culture and DNA

purification of positive clones, following the QUIAGEN plasmid purification protocol (Qiawell® Plasmid Purification System, Valencia, CA, USA), the MDR1 was sequenced.

#### **4.2.1.5 DNA Sequencing and Blast Analysis**

MDR1 gene sequencing was performed using ABI Prism 377 DNA Sequencer, Version 3.1.1 (Applied Biosystems, Foster, CA, USA). The partial MDR1 gene sequence obtained was aligned against the genomic NCBI database for homology comparison with the sequence of other species [Blast (Basic Local Alignment Search Tool) Analysis] (NCBI/BLAST, nucleotide blast: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### **4.2.2 PART 2. Expression of MDR1 Gene in Equine Tissues**

#### **4.2.2.1 Design of Primers and Probes**

Forward and reverse gene-specific primers and probe for MDR1 gene were designed using Primer Express™ 1.5 software (Applied Biosystem, Foster, CA, USA) and the partial DNA sequence of equine MDR1 obtained in the first part of this study<sup>29</sup>. Sequences of primers and probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), equine beta-actin, and 18 S genes were obtained from the literature (Table 4.2). Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) and used at final concentration of 20 uM. Probes were synthesized by Biosearch Technologies (Novato, CA, USA) and used at concentration of 10 pmoles/uL. The internal probe was labeled at the 5' end with the reporter dye FAM (6-carboxyfluorescein) and at the 3' end with Black Hole Quencher (BHQ).

#### **4.2.2.2 Template**

##### **4.2.2.2.1 Tissue Collection**

Samples of oral mucosa (between buccal commissure and cheek), sublingual (from the sublingual fold down to the floor of sublingual area), esophagus, stomach (glandular and



nonglandular parts), small intestine (duodenum -proximal and distal parts, jejunum, ileum), and liver were collected from 10 adult Thoroughbred, Quarter Horse, or Peruvian Paso horses (8 males and 2 females), 30 minutes after humane euthanasia for reasons of debilitation (orthopedic or reproductive problems) or donation. Horses ranged from 3 to 11 years of age (mean of 7.4 years) and were free of medication at least two-weeks prior to euthanasia. This study was approved by the Louisiana State University Institutional Animal Care and Use Committee.

**Table 4.2 – Design of Primers and Probes**

Gene	Accession Number (GenBank)	5'-3'	Sequence	Reference
MDR1	AY968084	Primer F Primer R Probe	CAGGAGCCCATCCTGTTTGA CACGACCCGGCTGTTGTC ATAGGCGATGTTCTCACCAATGCTGCA	Natalini <i>et al.</i> , 2005 <sup>38</sup>
GAPDH	AF097178	Primer F Primer R Probe	AAGTGGATATTGTCGCCATCAAT AACTTGCCATGGGTGGAATC TGACCTCAACTACATGGTCTACATGTTTCA	Leutenegger <i>et al.</i> , 1999 <sup>39</sup>
BETA-ACTIN	AF035774	Primer F Primer R Probe	AGGGAAATCGTGCGTGACA GCCATCTCCTGCTCGAAGTC CAAGGAGAAGCTCTGCTATGTCGCCCT	NCBI
18 S	AJ311673	Primer R Primer R Probe	AAACGGCTACCACATCCAA TCGGGAGTGGGTAATTTGC AAGGCAGCAGGCGC	Allen <i>et al.</i> , 2007 <sup>40</sup>

Primer F: Forward Primer, Primer R: Reverse Primer. NCBI: National Center for Biotechnology Information.

Table shows the gene accession numbers in the GenBank, designed sequences and literature references for forward and reverse primers and probes of equine MDR1, GAPDH, beta-actin, and 18 S genes.

#### 4.2.2.2.2 RNA Isolation

Total RNA from tissues was isolated following the TRI REAGENT<sup>®</sup> LS protocol. Potentially contaminating genomic DNA was digested with DNase I Amplification Grade (1,000 U – 1U/uL), 10X DNase I buffer solution, and 25 mM EDTA (Invitrogen, Carlsbad, CA, USA) at 65°C for 10 minutes. RNA concentration and purity were determined by spectrophotometry.

#### 4.2.2.2.3 cDNA Synthesis

Complementary DNA was synthesized by adding 2400 ng of total RNA of the samples to a master mix solution containing 5X first strand (FS) buffer, 10mM dNTP (dATP, dTTP, dGTP and dCTP), Oligo dT, 0.1 M DTT, RNasout (Recombinant Ribonuclease Inhibitor, 5,000 U - 40 U/uL), M-MLV-RT (moloney murine leukemia virus reverse transcriptase, 40,000 U - 200U/uL) (Invitrogen, Carlsbad, CA, USA), 1X BSA (BioLabs Ipswich, MA, USA), and DEPC treated water (Ambion, Austin, TX, USA). The reaction proceeded at 40°C for 1 hour and cDNA was used as the template for Real-Time PCR.

#### 4.2.2.3 Real-Time PCR (TaqMan)

Real-Time PCR was performed by 7900HT Sequence Detection System version 2.3 (Applied Biosystems, Foster, CA, USA) under ideal conditions (50°C-2 min, 95°C-10 min, 40 cycles of 95°C-15 sec and 60°C-1 min). The reaction was carried out in duplicates in a 384 well plate containing cDNA (template), TaqMan Universal PCR Master Mix (Applied Biosystem, Foster, CA, USA), forward and reverse primers and an internal oligonucleotide as a probe. Relative quantitation of MDR1 gene expression was estimated using the relative standard curve method and reported as the fold-difference in nucleic acid across tissues. Values of MDR1 gene to match the threshold cycle (Ct) were calculated using the equation that describes the relationship between the Ct and the curve values. MDR1 gene values were normalized to the

housekeeping gene and comparison across tissues was performed by designing a specific normalized sample as a calibrator or reference tissue (oral mucosa).

#### 4.2.3 PART 3. Expression of P-glycoprotein

In order to investigate the expression of P-gp in horse tissues, the partial sequence of the equine MDR1 gene obtained in the first part of this study was cloned and induced into pGEX-6P-1 plasmid vector (GST gene fusion system, Amersham Biosciences, Piscataway, NJ, USA) to serve as our positive control.

Plasmid pcDNA3.1/MDR1 was used as the template for the PCR reaction which was performed under ideal conditions (denaturation: 94°C – 30 seconds, annealing: 62°C – 30 seconds, and extension: 72°C – 1 minute, 33 cycles). Forward and reverse primers used for the PCR reaction comprised 20 nucleotide base pairs of the gene of interest (MDR1, GenBank: AY968084)<sup>38</sup>, BamHI or EcoRI restriction enzymes, stop codon (Reverse primer), and six extra nucleotides (Table 4.3). Amplification specificity was determined by agarose electrophoresis. After DNA purification by precipitation using 5 M potassium acetate and isopropanol, PCR product (amplified DNA) was digested with BamHI and EcoRI for subsequent cloning.

**Table 4.3 – Design of Forward and Reverse Primers (GenBank AY968084)**

Primer	Sequence
Forward 5' - 3'	GCAGAC <u>CGGATT</u> <b>CAAAGCCAAAGTGT</b> CAGCAGC
Reverse 5' - 3'	GTCTGCGA <u>ATTCT</u> CACACAATGCAGGTGCGGCCTT

Table shows the designed sequences for forward and reverse primers using the horse MDR1 gene partial sequence obtained in the first part of this study (*Equus caballus* multi-drug resistance P-glycoprotein 1 (MDR1) mRNA, partial sequence cds. GenBank AY968084). Sequences in bold correspond to the beginning (Forward primer) and end (Reverse primer) of the equine MDR1 gene partial sequence. Underline sequences correspond to BamHI (Forward primer) and EcoRI (Reverse primer) restriction enzymes since plasmid pcDNA3.1/MDR1 was used as the template. Nucleotide triplet in italic corresponds to the stop codon in the Reverse primer.

#### 4.2.3.1 Plasmid pGEX-6P-1/MDR1

MDR1 gene was cloned into pGEX-6P-1 vector, containing the glutathione-S-transferase [GST] tag (Amersham Biosciences, Piscataway, NJ, USA), and chemically transformed into *Escherichia coli* competent cells (One Shot TOP10F, Invitrogen, Carlsbad, CA, USA). After culture and DNA purification (QUIAGEN Plasmid Purification System), positive clones were sequenced.

After sequencing, MDR1 gene cloned into pGEX-6P-1 (pGEX-6P-1/MDR1) or pGEX-6P-1 vector alone were transformed into BL21 *Escherichia coli* cells (OneShot® BL21 Star™, Invitrogen, Carlsbad, CA, USA) and grown in Luria-Bertani (LB) medium with ampicillin (100 µg/mL) for approximately 2.5 h until the optical density at 600 [OD<sub>600</sub>] (absorbance at a wavelength of 600nm) of the culture reaches 0.6. Isopropyl-β-D-thiogalactoside [IPTG] (Gold Biotechnologies, St. Louis, MO, USA) at a final concentration of 0.5mM was added to the culture to induce expression, followed by 1 h of culturing at 37°C at a shaking rate of 250 rpm. Bacterial cells were harvested by centrifugation (7,7 xg, 10 min, 4°C) and stored at -20°C until analysis. Protein expression was visualized and confirmed by coomassie brilliant blue staining (Bio-Rad Laboratories, Hercules, CA, USA) and western blot analysis after sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE).

#### 4.2.3.2 Western Blot

Harvest bacterial cells were lysed at room temperature for 30 min using the bacterial protein extraction reagent (B-PER®, Pierce, Rockford, IL, USA) with 20 mg/L lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor cocktail (Invitrogen, Carlsbad, CA, USA). P-gp-GST fusion protein was used as positive control for western blot analysis of proteins isolated from horse tissues.

Cells from the oral mucosa, sublingual, duodenum proximal and distal, jejunum, ileum, and liver tissues of horses were lysed using mammalian protein extraction reagent (M-PER<sup>®</sup>, Pierce, Rockford, IL, USA), as per the manufacturers' instructions.

All samples were diluted in a  $\beta$ -mercaptoethanol containing sample loading buffer, heated at 98°C for 5 min and electrophoretically separated in a 4 to 20% SDS-polyacrylamide gel (precise protein gel, Pierce, Rockford, IL, USA) for 1 h at 98 volts. Specific molecular marker for western blot (Precision Plus Protein<sup>®</sup>WesternC<sup>™</sup>Standards, Bio-RAD, Hercules, CA, USA) was run in parallel with the samples. Following separation, proteins were transferred to a 0.45  $\mu$ m nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA) for 1 h at 33 volts. Membranes were blocked against nonspecific binding using 5% nonfat dry milk in 0.1% Tween-20 PBS - PBST overnight at 4°C. After washed three times for 15 min each with 0.1% PBST, membranes were incubated with the primary antibody mouse monoclonal to P-gp [C219] (diluted 1:400 in PBST) or [C494] (diluted 1:1,000 in PBST) for 1 h at room temperature. Following three washes (15 min each), membranes were then incubated with the secondary goat polyclonal to mouse IgG – H&L (Horseradish Peroxidase-HRP) antibody (diluted 1:20,000 in PBST) and 1X marker conjugate (Precision Protein Streptactin-HRP Conjugate, Bio-RAD, Hercules, CA, USA) for 1 h at room temperature and washed three more times. Blots and marker were visualized by chemiluminescence (ChemiDoc Gel Quantitation System using, Bio-Rad, Hercules, CA, USA) using the ELC<sup>™</sup> western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) as per the manufacturer's instructions. All primary C219 (ab3364), C494 (ab3365) and secondary (ab6789) antibodies were obtained from Abcam Inc. (Cambridge, MA, USA).

The GST protein (vector alone) and our positive control P-gp-GST fusion protein were previously confirmed by western blot using the primary monoclonal antibody mouse anti-GST (Invitrogen, Carlsbad, CA, USA).

#### **4.2.4 Statistical Analysis**

Gene identification and sequencing, and protein expression were reported as a descriptive analysis. Values of MDR1 gene normalized for the housekeeping GAPDH gene were analyzed by SAS 9.1 using ANOVA and Tukey as a post hoc test for comparison of mRNA expression levels of MDR1 across tissues with significance set at  $P < 0.05$ . Fold-difference of mRNA MDR1 between tissues was compared against the oral mucosa tissue used as a calibrator or reference tissue.

### **4.3 Results**

#### **4.3.1 Identification, Sequencing, and Blast Analysis of Equine MDR1 Gene**

An extensive search in the genomic database (Blast -Basic Local Alignment Search Tool-analysis) performed at the beginning of this study did not identify any partial or complete sequence for the MDR1 gene in the equine species.

From all three forward (F) and reverse (R) primers designed from rabbit, only primers in the positions 3113–3137 (F) and 3903–3879 (R) were able to identify similar sequence on the horse DNA. Positive PCR products were observed in a 1% agarose gel as single and discrete bands at correct size about 700 base pairs (bp). The partial sequence of the equine MDR1 gene was successfully cloned into the pcDNA 3.1 plasmid vector and confirmed by restriction digestion and agarose electrophoresis.

The DNA fragment sequenced corresponded to a partial sequence of 692 base pairs of the equine MDR1 gene. Sequence was analyzed for correct orientation and aligned with the rabbit

gene sequence for homology comparison. The alignment between equine MDR1 partial and ABCB1 rabbit DNA sequences revealed 84% of identity. Blast analysis against the genomic NCBI database showed also high similarity of equine MDR1 gene with other species including canine and feline (nucleotide blast: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The partial sequence of the equine MDR1 gene was first published in the NCBI GenBank – PubMed (Accession number: AY968084) on March 2005 (Figure 4.1)<sup>38</sup> and this sequence was subsequently used for further studies. Only in 2007, the predicted equine MDR1 gene sequence was published in the NCBI GenBank, and it was modified in 2008 (XM\_001492023, PREDICTED: *Equus caballus* multi-drug resistance p-glycoprotein 1 (MDR1), mRNA, 4785 bp). This sequence is derived from a genomic sequence of the *Equus caballus* (domestic horse) Genome Project (*Equus caballus* chromosome 4 genomic contig, reference assembly [based on EquCab2], whole genome shotgun sequence, 93951 bp, GenBank: NW 001867413). The alignment of the partial and predicted sequences of the equine MDR1 gene showed 99% of identity (Figure 4.2).

#### 4.3.2 Differential MDR1 mRNA Expression in Equine Tissues

Real-Time PCR efficiencies were calculated using a relative standard curve derived from a serial pooled DNA mixture (a 10-fold dilution series with six measuring points) obtained from the jejunum and ileum tissues collected from four young and old horses. GAPDH, beta-actin, and 18 S genes were analyzed and log transformed to meet the requirement of normal distribution for statistical analysis. Among them, GAPDH was the most stable gene with the best PCR efficiency. GAPDH was used as the housekeeping gene for normalization of the MDR1 mRNA expression levels in horse tissues in this study (Table 4.4).

AY968084.1	
Equus caballus (horse) partial multi-drug resistance p-glycoprotein 1	
Equus caballus (horse)	
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Laurasiatheria; Perissodactyla; Equidae; Equus.	
NCBI_TaxID= <a href="#">9796</a> ;	
Key	Location/Qualifiers
<a href="#">source</a>	1..692 /organism="Equus caballus" /mol_type="mRNA"
<a href="#">CDS</a>	AY968084.1:<1..>692 /codon_start=1 /gene="MDR1" /product="multi-drug resistance p-glycoprotein 1" /db_xref=" <a href="#">GOA:Q56B93</a> " /db_xref=" <a href="#">InterPro:IPR003439</a> " /db_xref=" <a href="#">InterPro:IPR003593</a> " /db_xref=" <a href="#">InterPro:IPR017871</a> " /db_xref=" <a href="#">UniProtKB/TrEMBL:Q56B93</a> " /protein_id=" <a href="#">AAX78354.1</a> " /translation="KAKVSAAHIIIMIEKTPLIDSYSTEGLKPNTLEGNVIFNEVVFNY PTRPDIPVLQGLSVEVKKQTALVGS SGGCKSTLVQLLERFYDPMAGTVLLDGTEIKH LNVQWLR AHLGIVSQEPILFDCSIGENIAYGDN SRVVSQEEIVQAAKEANIHPFIETLP DKYNTRVGDKGTQLSGGQKQRIAIARALVRQPQILLLDEATSALDTESEKVVQEALDKA REGRTICIV"
Sequence 692 BP; 197 A; 172 C; 184 G; 139 T; 0 other; 1001075923 CRC32;	
aaagccaaag tgtcagcagc ccacatcatc atgatcattg aaaaaacccc tctgatcgac 60 agctatagca cagaaggcct aaagccaaat acattggagg gaaatgtgat ctttaatgaa 120 gttgtgttca actatccac tcgaccagac atccagtgct ttcaggggct gacggttgag 180 gtaaagaagg gccagacgct cgccctggtg ggcagcagtg gctgtgggaa gacacactg 240 gtccagctcc ttgagcgtt ctatgacccc atggcgggaa cagtgttact tgacggcaca 300 gaaataaagc acctgaatgt ccagtggctc cgagcacacc tgggcattgt gtcccaggag 360 cccacacctg ttgactgcag cattggtgag aacatgcct atggagacaa cagccgggtc 420 gtgtcacagg aagagattgt gcaggcagcc aaggaggcca acatacacc cttcatcgag 480 acactgcctg ataaatataa caccagagta ggagacaaag gaactcagct ctctggtggc 540 cagaaacagc gtattgcgat agctcgggcc cttgtgagac agcctcagat tttgcttttg 600 gatgaagcta catcagctct ggatacagaa agtgaaaagg ttgtccaaga agctctggac 660 aaagccagag aaggccgcac ctgcattgtg at 692	

**Figure 4.1 – *Equus caballus* Multidrug Resistance P-glycoprotein (MDR1) mRNA, Partial cds**

Partial sequence of the equine MDR1 gene published in the National Center for Biotechnology Information (NCBI), GenBank on PubMed (Accession number: AY968084)<sup>38</sup>.



```

Basic Local Alignment Search Tool (BLAST)

Query: AY968084.1 - Equus caballus multi-drug resistance p-glycoprotein 1 (MDR1) mRNA, partial cds
(nucleic acid, 692bp)

Subject: XM_001492023.2 - PREDICTED: Equus caballus multi-drug resistance p-glycoprotein 1
(MDR1), mRNA (nucleic acid, 4785bp)

Score = 1251 bits (677), Expect = 0.0
Identities = 687/692 (99%), Gaps = 0/692 (0%)
Strand=Plus/Plus

Query 1 AAAGCCAAAGTGTCTAGCAGCCACATCATGATCATTGAAAAACCCCTCTGATCGAC 60
      |||
Sbjct 3943 AAAGCCAAAGTGTCTAGCAGCCACATCATGATCATTGAAAAACCCCTCTGATCGAC 4002

Query 61 AGCTATAGCACAGAAGGCCTAAAGCCAAATACATTGGAGGGGAAATGTGATCTTTAATGAA 120
      |||
Sbjct 4003 AGCTATAGCACAGAAGGCCTAAAGCCAAATACATTGGAGGGGAAATGTGATCTTTAATGAA 4062

Query 121 GTTGTGTTCACCTATCCCACTCGACAGACATCCCACTGCTTCAGGGGCTGAGCGTTGAG 180
      |||
Sbjct 4063 GTTGTGTTCACCTATCCCACTCGACAGACATCCCACTGCTTCAGGGGCTGAGCGTTGAG 4122

Query 181 GTAAAGAAGGGCCAGACGCTCGCCCTGGTGGGCAGCAGTGGCTGTGGGAAGAGCACACTG 240
      |||
Sbjct 4123 GTAAAGAAGGGCCAGACGCTCGCCCTGGTGGGCAGCAGTGGCTGTGGGAAGAGCACACTG 4182

Query 241 GTCCAGCTCCTTGAGCGGTTCTATGACCCCATGGCCGGAACAGTGTACTTGACGGCACA 300
      |||
Sbjct 4183 GTCCAGCTCCTTGAGCGGTTCTATGACCCCATGGCCGGAACAGTGTACTTGACGGCACA 4242

Query 301 GAAATAAAGCACCTGAATGTCCAGTGGCTCCGAGCACACCTGGGCATTGTGTCCAGGAG 360
      |||
Sbjct 4243 GAAATAAAGCACCTGAATGTCCAGTGGCTCCGAGCACACCTGGGCATTGTGTCCAGGAG 4302

Query 361 CCCATCCTGTTTGACTGCAGCATTGGTGAGAACATCGCCTATGGAGACAACAGCCGGGTC 420
      |||
Sbjct 4303 CCCATCCTGTTTGACTGCAGCATTGGTGAGAACATCGCCTATGGAGACAACAGCCGGGTC 4362

Query 421 GTGTCACAGGAAGAGATTGTGAGGCAGCCAAAGGAGGCCAACATACACCCCTTCATCGAG 480
      |||
Sbjct 4363 GTGTCACAGGAAGAGATTGTGAGGCAGCCAAAGGAGGCCAACATACACCCCTTCATCGAG 4422

Query 481 ACACCTGCCTGATAAATAACACCCAGAGTAGGAGACAAAGGAACTCAGCTCTCTGGTGGC 540
      |||
Sbjct 4423 ACACCTGCCTGATAAATAACACCCAGAGTAGGAGACAAAGGAACTCAGCTCTCTGGTGGC 4482

Query 541 CAGAAACAGCGTATTGCGATAGCTCGGCCCTTGTGAGACAGCCTCAGATTTTGCTTTTG 600
      |||
Sbjct 4483 CAGAAACAGCGTATTGCGATAGCTCGGCCCTTGTGAGACAGCCTCAGATTTTGCTTTTG 4542

Query 601 GATGAAGCTACATCAGCTCTGGATACAGAAAGTGAAAAGGTTGTCCAAGAAGCTCTGGAC 660
      |||
Sbjct 4543 GATGAAGCTACATCAGCTCTGGATACAGAAAGTGAAAAGGTTGTCCAAGAAGCTCTGGAC 4602

Query 661 AAAGCCAGAGAAGGCCGACCTGCATTGTGAT 692
      |||
Sbjct 4603 AAAGCCAGAGAAGGCCGACCTGCATTGTGAT 4634

```

**Figure 4.2 – BLAST (Basic Local Alignment Search Tool) Analysis Between Partial and Predicted Sequences of the Equine Multidrug Resistance Gene (MDR1) mRNA**

The alignment shows 99% of identity between partial sequence obtained in this study (Accession number: AY968084) and the predicted sequence (Accession number: XM\_001492023) of the equine MDR1 gene. Predicted sequence is derived from a genomic sequence of the *Equus caballus* (domestic horse) Genome Project (*Equus caballus* chromosome 4 genomic contig, reference assembly [based on EquCab2], whole genome shotgun sequence, 93951 bp, GenBank: NW 001867413). Both partial and predicted gene sequences are published in the National Center for Biotechnology Information (NCBI), GenBank on PubMed.

**Table 4.4 – Real-Time PCR Reaction Efficiency (%)**

<b>Genes</b>	<b>R-squared</b>	<b>Slope</b>	<b>Efficiency (%)</b>
MDR1	0.999	-3.6257	88.7
GAPDH	0.999	-3.8313	82.4
Beta-actin	0.999	-4.0718	76.0
18 S	0.967	-4.0055	77.7

Table shows the R-squared, slope, and efficiency of Real-Time PCR (TaqMan) reaction for the gene of interest MDR1 and each housekeeping genes GAPDH, beta-actin and 18 S.

To compare the expression levels of the MDR1 gene across different tissues in horses, gene expression was calculated from the Ct values of samples based on the PCR efficiency equation determined by the standard curve analysis. Expression of MDR1 gene was normalized for the housekeeping GAPDH gene. The MDR1 mRNA expression level was significantly higher in the stomach (glandular portion), all three parts of the small intestine, and liver. Relative quantitation of MDR1 gene expression was reported as the fold difference of nine different tissues compared against the oral mucosa chosen as the reference tissue or calibrator (Table 4.5, Figure 4.3). Expression of the MDR1 gene was approximately 10-fold higher in the glandular portion of stomach (SGI) and 20-fold higher in the liver (Liv) when compared to the oral mucosa (OM). In the small intestine, MDR1 gene expression was more than 20-, 40-, 130-, and 180-fold higher than oral mucosa in the proximal (DProx) and distal (DDist) parts of the duodenum, jejunum (Jej), and ileum (IL), respectively (overall P value = 0.0056). Gene expression in the sublingual (Sub), esophagus (Eso) and nonglandular portion of the stomach (SNon) tissues was not significantly different compared to the expression of MDR1 gene in the oral mucosa tissue.

**Table 4.5. Fold-Difference of Relative Quantitation of MDR1 mRNA Expression in Equine Tissues**

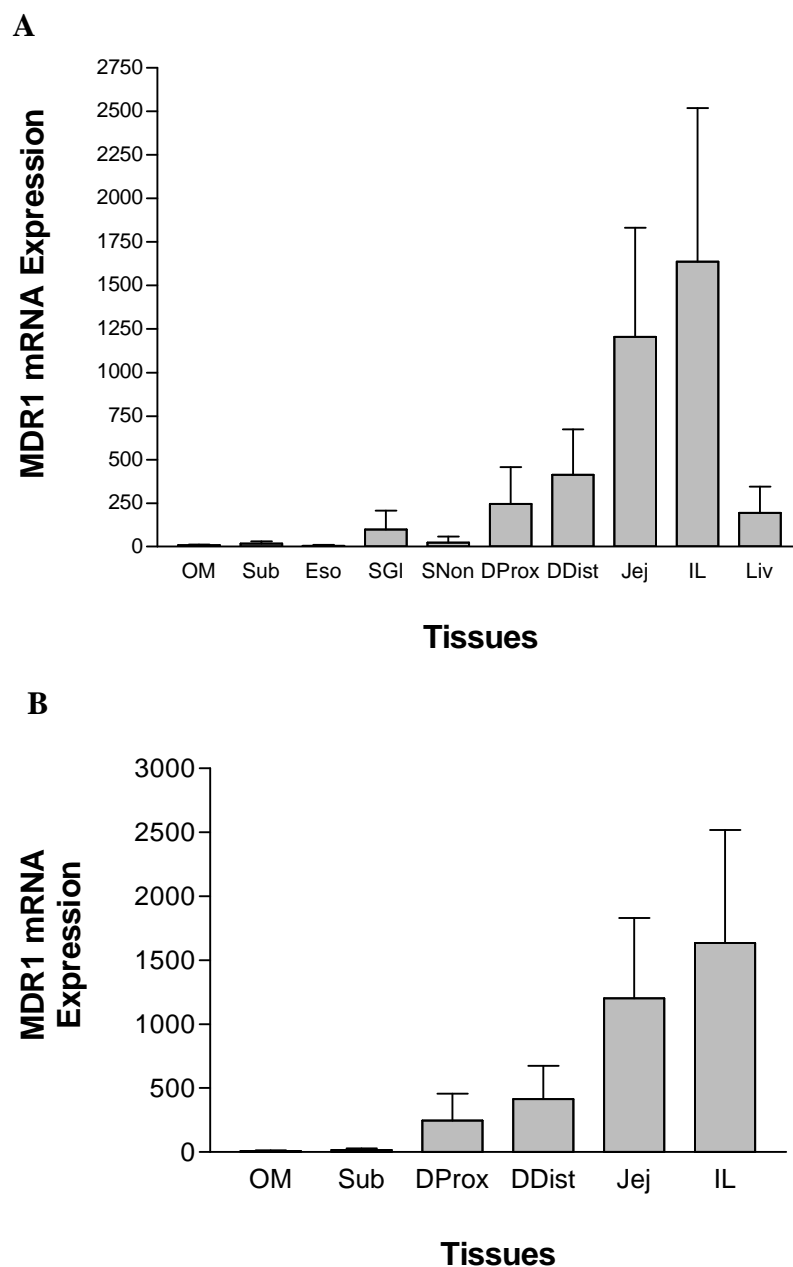
TISSUE	MDR1/GAPDH VALUES	FOLD DIFFERENCE (P=0.0056)
<b>OM</b>	8.69 $\pm$ 3.9	Calibrator
<b>Sub</b>	17.19 $\pm$ 12.4	1.98
<b>Eso</b>	5.24 $\pm$ 4.4	0.6
<b>SGI</b>	97.72 $\pm$ 109.6	11.25
<b>SNon</b>	23.7 $\pm$ 34.7	2.73
<b>DProx</b>	245.49 $\pm$ 211.0	28.2
<b>DDist</b>	412.87 $\pm$ 261.5	47.5
<b>Jej</b>	1203.32 $\pm$ 628.6	138.5
<b>IL</b>	1635.71 $\pm$ 884.1	188.2
<b>Liv</b>	194.34 $\pm$ 150.9	22.4

OM – oral mucosa (reference tissue/calibrator), Sub – sublingual, SGI – stomach (glandular part), SNon – stomach (nonglandular part), DProx – proximal segment of duodenum, DDist – distal segment of duodenum, Jej – jejunum, IL – ileum, Liv – liver.

Table represents the values  $\pm$ SD of MDR1 gene expression levels normalized to GAPDH gene and fold-difference across equine tissues including sublingual, esophagus, stomach, small intestine, and liver compared to the oral mucosa. Oral mucosa was used as a calibrator or reference tissue.

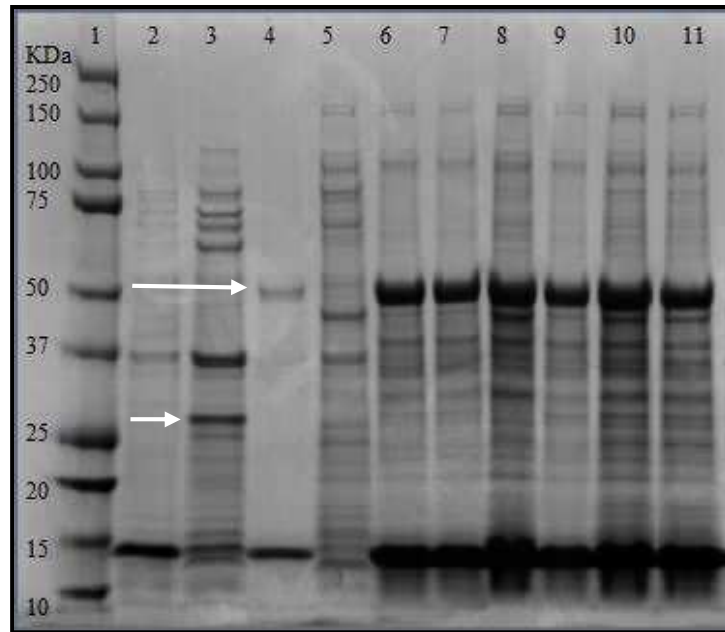
#### 4.3.3 Expression of Equine P-glycoprotein

The partial sequence of the equine MDR1 gene was successfully cloned into p-GEX-6P-1 plasmid vector and P-gp-GST fusion protein was effectively induced by 0.5 mM of IPTG at 37°C. It was confirmed by coomassie blue staining and western blot analysis using two primary antibodies. Induced glutathione-S-transferase (GST) protein alone corresponds to a size of 26 kDa and was used as a positive control for induced P-gp. The P-gp-GST fusion protein corresponded to a size of approximately 50 kDa (Figure 4.4, 4.5). P-gp was also recognized in all tissues evaluated (oral mucosa, sublingual, duodenum (proximal and distal), jejunum, ileum, and liver) using two primary monoclonal antibodies C219 and C494 (Figure 4.6).



**Figure 4.3 – Relative Quantitation of MDR1 mRNA Expression in Equine Tissues.**

The data represents the relative quantitation of MDR1 mRNA expression levels normalized with GAPDH gene in the oral mucosa (OM), sublingual (Sub), esophagus (Eso), glandular (SGI) and nonglandular (SNon) stomach, duodenum (proximal –Dprox and distal –Ddist), jejunum (Jej), ileum (IL), and liver (Liv) tissues. All values reflect the mean and  $\pm$  standard deviation of ten young and adult, male and female horses ( $P < 0.05$ ). Figure A compiles all tissues tested and figure B compares tissues possibly involved in the absorption process of oral administered drugs.



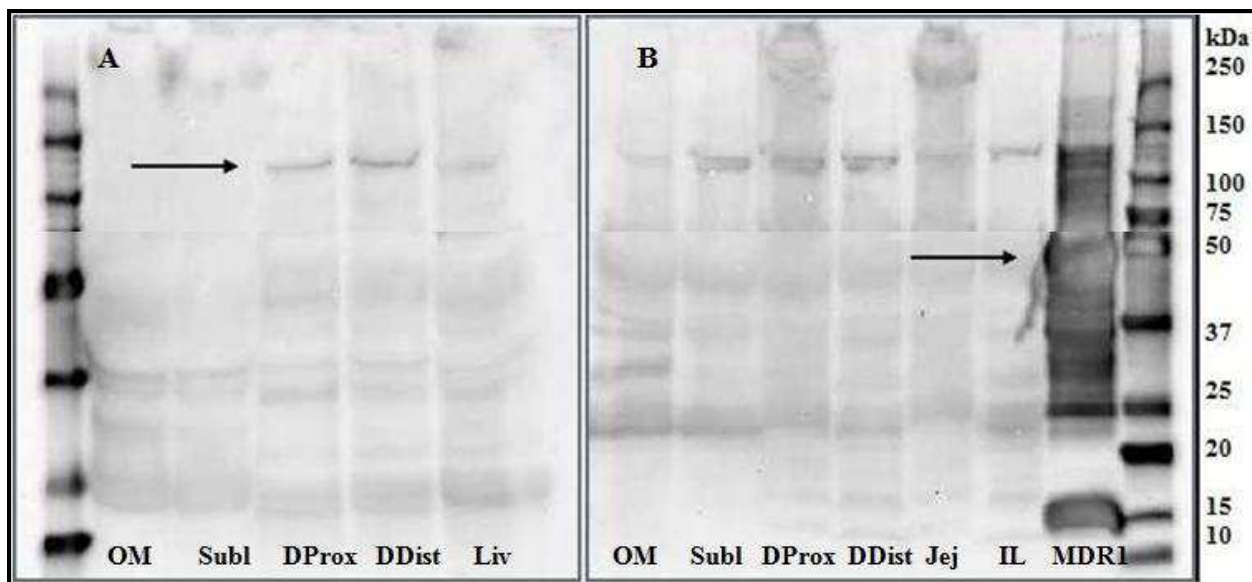
**Figure 4.4 – Coomassie Brilliant Blue Staining (SDS-PAGE).**

The coomassie blue stained SDS gel shows induced GST protein and P-gp-GST fusion protein in pGEX vector (white arrows). Lane 1 – marker, lanes 2 and 3 – non-expressed and expressed GST (approximately 26 kDa), lane 5 – non-expressed P-gp, Lanes 4 and 6 to 11 – induced P-gp (P-gp-GST fusion protein of approximately 50kDa).



**Figure 4.5 – Western Blot Analysis (P-gp-GST fusion protein).**

Western blot shows the GST protein [26 kDa] (A) and validates the expression of P-gp-GST fusion protein [50 kDa] (B) using an anti-GST primary antibody.



**Figure 4.6 – Western Blot Analysis (Expression of P-gp in Equine Tissues).**

Western blot indicating expression of P-gp in different tissues in horses including oral mucosa (OM), sublingual (Subl), proximal (DProx) and distal (DDist) duodenum, jejunum (Jej), ileum (IL), and liver (Liv) using C219 (A) and C494 (B) antibodies. P-gp in pGEX (P-gp-GSTfusion protein, 50 kDa) was recognized by both antibodies and used as a control for the expression of P-gp in tissues.

#### 4.4 Discussion

Previously to this study, an extensive search confirmed no sequence of equine MDR1 gene available in the National Center for Biotechnology Information – NCBI, GenBank and lack of published data related to MDR1 gene and P-gp in horses. The horse genome had not yet been completed by the time the partial sequence of equine MDR1 gene was accomplished in our laboratory, which was the first information about this gene in horses published in the NCBI GenBank – PubMed (Accession number: AY968084) on March 2005. The predicted equine MDR1 gene sequence (XM\_001492023, PREDICTED: *Equus caballus* multi-drug resistance p-glycoprotein 1 (MDR1), mRNA) was later published at the NCBI website in June 2007 and modified on July 2008 according to the NCBI GenBank – PubMed website. This sequence derived from a genomic sequence of the *Equus caballus* (domestic horse) Genome Project.

Due to similarities in the diet and digestion between horses and rabbits, the rabbit ABCB1 (MDR1) sequence was chosen for primer design to identify and amplify the equine MDR1 gene using PCR technique. High homology (77% to 99%) observed with the alignment between the partial sequence of equine MDR1 gene and the sequence of other species validated the authenticity of the equine partial sequence. The comparison between the partial and predicted sequences showed 99% identity at the positions 3943–4634 of the predicted equine MDR1 sequence (Identities = 687/692 (99%), Gaps = 0/692 (0%); alignment by BLAST).

Expression of MDR1 gene was confirmed in the oral cavity, esophagus, stomach, small intestine, and liver of horses using Real-Time PCR technique. The efficiency of the PCR reaction was estimated by the slope of a standard curve, graphically represented as a semi-log regression line plot of Ct value vs. log of input nucleic acid. Reaction efficiency corresponds to the rate at which a PCR amplicon (DNA sequence as the product of PCR amplification) is generated. Due

to the highest efficiency compared to Beta-actin and 18 S genes, the GAPDH gene showed was used as housekeeping gene which is constitutively expressed in most tissues, to normalize the mRNA expression of MDR1 gene. Compared to the oral mucosa, MDR1 mRNA expression was significantly higher in the small intestine of horses, increasing from proximal to distal segments (duodenum < jejunum < ileum). The small intestine, in particular duodenum and jejunum segments, is the potential site for drug absorption after *in vivo* oral administration; therefore, high expression of MDR1 gene in these tissues could limit intestinal absorption and decrease drug bioavailability in horses. In the other hand, low gene expression of MDR1 in the oral mucosa may favor drug absorption through the oral cavity after *in vivo* oral drug administration. *In Vivo* studies performed in our laboratory demonstrated high serum concentration of methadone after oral drug administration to horses<sup>41</sup>, but higher oral compared to intestinal bioavailability (unpublished data).

As reported in other species, the MDR1 gene is highly expressed in the small intestine of horses and its expression increases from the proximal to the distal segments. When compared to the proximal duodenum, the MDR1 gene was approximately 2-, 5-, and 7-fold higher in the distal duodenum, jejunum, and ileum, respectively. Studies in humans, mice, and rats showed similar patterns for MDR1 mRNA expression levels in the small intestine, increasing progressively from proximal to distal regions. In humans, Zimmermann *et al.* (2005) reported a 4-fold higher expression of MDR1 in the terminal ileum compared with the duodenum. As well in rats and mice, the highest level of *mdr3* (or *mdr1a*) expression was reported in the ileum, when compared to the duodenum<sup>4,42-45</sup>.

The expression and location of MDR1 gene and P-gp were recently investigated in the intestine, liver, kidney and lymphocytes of horses, showing conflicting results to our findings.



According to Tyden *et al.* (2008), comparing MDR1 gene expression in different segments of the small and large intestine demonstrated higher gene expression in the distal portion of duodenum and proximal portion of jejunum. Levels of gene expression were decreased toward the distal jejunum, ileum, cecum, and colon in horses, different from our results that showed higher levels in the jejunum and ileum. These authors suggested no significant correlation between mRNA and protein expression of P-gp in the various intestinal segments<sup>45</sup>.

The presence of P-gp in different examined horse tissues was confirmed by western blot analysis using P-gp-GST fusion protein as a positive control. Glutathione-S-transferase (GST) is a tag or fusion protein in the pGEX expression vector used to detect and purify target proteins. MDR1 gene was clone into vector pGEX-6P-1 inframe with the GST gene at the N-terminal; therefore, the induction resulted in expression of the protein of interest (P-gp) fused to the GST protein. P-gp correspond to approximately 25 kDa and after fused with GST (26 kDa), P-gp-GST fusion protein was about 50 kDa. Expression and sizes were confirmed by coommasie blue staining and western blot analysis using an anti-GST antibody. The western blot indicated the presence of P-gp in all examined tissues, oral mucosa, sublingual, proximal and distal duodenum, jejunum, ileum, and liver using two monoclonal primary antibodies C219 and C494. Bands were observed with a size of approximately 150 kDa in agreement with Tyden *et al.* (2008) who previously identified P-gp in equine tissues with a size identical to that of human recombinant (approximately 150 kDa)<sup>45</sup>. Both monoclonal antibodies used in this study are able to recognize two different and specific internal cellular epitopes. Antibody C219 recognizes a highly conserved amino acid sequence VQEALD and C494 detects the amino acid sequence PNTLEGN, both present in the partial sequence of equine MDR1 gene.

Determining the expression of MDR1 gene and P-gp in different tissues was the first step to investigate transporter proteins in horses. However, the presence of the gene or expression of the protein does not necessarily provide information about protein function. Additional studies are necessary to determine the role of MDR1-P-gp in the absorption and oral pharmacokinetics of opioids in horses.

#### 4.5 References

1. Martinez, M., Modric, S., Sharkey, M. et al. (2008) The pharmacogenomics of P-glycoprotein and its role in veterinary medicine. *Journal of Veterinary Pharmacology and Therapeutics*. 31, 285-300.
2. Zhou, S. F. (2008) Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*. 38(7-8), 802-832.
3. Brady, J. M., Cherrington, N. J., Hartley, D. P., Buist, S. C., Li N., Klaassen, C. D. (2002) Tissue distribution and chemical induction of multiple drug resistance genes in rats. *Drug Metabolism and Disposition*. 30(7), 838-844.
4. Zimmermann, C., Gutmann, H., Hruz, P. Gutzwiller, J-P., Beglinger, C., Drewe, J. (2005) Mapping of multidrug resistance gene 1 and multidrug resistance associated protein isoform 1 to 5 mRNA expression along the human intestinal tract. *The American Society for Pharmacology and Experimental Therapeutics*. 33(2), 219-224.
5. Wang, J. S., Yiang, R., Taylor, R. M. et al. (2004) Brain penetration of methadone (R)- and (S)-enantiomers is greatly increased by P-gp deficiency in the blood-brain barrier of Abcb1a gene knockout mice. *Psychopharmacologia*. 173(1), 132-8.
6. Thompson, S. J., Koszdin, K., Bernards, C. B. (2000) Opioid-induced analgesia is increased and prolonged in mice lacking P-glycoprotein. *Anesthesiology*. 92(5), 1392-1399.
7. Sun, J., He, Z. G., Chen, G. et al. (2004) Multidrug resistance P-glycoprotein: crucial significance in drug disposition and interaction. *Medical Science Monitor*. 10(1), RA5-14.
8. Katsura, T., Inui, K-I. (2003) Intestinal absorption of drugs mediated by drug transporters: Mechanisms and Regulation. *Drug Metab. Pharmacokin*. 18(1), 1-15.
9. Marchetti, S., Mazzant, R., Beijnen, J. H., Schellens, J. H. M. (2007) Clinical relevance of drug-drug and herb-drug interactions mediated by the ABC Transporter ABCB1 (MDR1, P-glycoprotein). *The Oncologist*, 12, 927-941.

10. Fardel, O., Lecureur, V., Guillouzo, A. (1996) The P-glycoprotein multidrug transporter. *Gen. Pharmac.*, 27(8), 1283-1291.
11. Cascorbi, I. (2006) Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. *Pharmacology & Therapeutics*, 112, 547-473.
12. Fromm, M. F. (2004) Importance of P-glycoprotein at blood-tissue barriers. *TRENDS in Pharmacological Sciences*. 25(8), 423-429.
13. Matheny, C. J., Lamb, M. W., Brouwer, K. L. R., Pollack, G. M. (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy*, 21(7), 778-796.
14. Kimura, Y., Morita, S., Matsuo, M., Ueda, K. (2007) Mechanism of multidrug recognition by MDR1/ABCB1. *Cancer Science*. 98(9), 1303-1310.
15. Di Pietro, A., Dayan, G., Conseil, G. et. al. (1999) P-glycoprotein-mediated resistance to chemotherapy in cancer cells: using recombinant cytosolic domains to establish structure-function relationships. *Brazilian Journal of Medical and Biological Research*. 32, 925-939.
16. Juliano, R. L., Ling, V. (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta*, Amsterdam. 455, 152-162.
17. Mealey, K. L. (2004) Therapeutic implications of the MDR-1 gene. *Journal of Veterinary Pharmacology and Therapeutics*. 27, 257-264.
18. Ieiri, I., Takane, H., Otsubo, K. (2004) The *MDR1* (ABCB1) gene polymorphism and its clinical implications. 49(9), 553-576.
19. Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Briedler, J. L., Melamed, M. R., Bertino, J. R. (1989) Multidrug resistance gene is expressed by endothelial cells at blood-brain barrier sites. *Proceedings Nat. Acad. Sci. of USA*. 86, 695-698.
20. Mizuno, N., Niwa, T., Yotsumoto, Y., Sugiyama, Y. (2003) Impact of drug transporter studies on drug discovery and development. *Pharmacological Reviews*. 55(3), 425-461.
21. Thiebaut, F., Tsuruo, T., Gottesman, M. M., Pastan, I., Willingham, M. C. (1987) Cellular localization in normal human tissues. *Proceedings of the National Academy of Sciences of USA*. 84, 7735-7738.
22. Schinckel, A. H., Smit, J. J., Telling, O., Beijnen, J. H., Wagenaar, E. et al. (1994) Disruption of the Mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell*. 77, 491-502.
23. Dagenais, C., Graff, C. L., Pollack, G. M. (2004) Variable modulation of opioid brain uptake by P-glycoprotein in mice. *Biochemical Pharmacology*. 67, 269-276.

24. Xie, R., Hammarlund-Udenaes, M., de Boer, A. G., de Lange, E. C. M. (1999) The role of P-glycoprotein in blood-brain barrier transport of morphine: transcortical microdialysis studies in *mdr1a* (-/-) and *mdr1b* (+/+) mice. *British Journal of Pharmacology*. 128, 563-568.
25. Cummins, C. L., Salphati, L., Reid M. J., Benet, L. Z. (2003) In vivo modulation of intestinal CYP3A metabolism by P-glycoprotein: Studies using the rat single-pass intestinal perfusion model. *Journal of Pharmacology and Experimental Therapeutics*. 305(1), 306-314.
26. Mukhopadhyay, T., Batsakis, J. G., Kuo, M. T. (1998) Expression of the *mdr* (P-glycoprotein) gene in Chinese hamster digestive tracts. *Journal of the National Cancer Institute*. 80(4), 269-275.
27. Collaghan, R., Riordan, J. R. (1993) Synthetic and natural opiates interact with P-glycoprotein in multidrug-resistant cells. *The Journal Biological Chemistry*. 268(21), 16050-16064.
28. Schinkel A. H, Wagenaar E., Mol C. A. A. M., Deemter L. V. (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation*. 97, 2517-2524.
29. Sparreboom, A., van Asperen, J., Mayer, U., Schinckel, A. H. et al. (1997) Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proceedings of the National Academy of Sciences of the United States of America*. Washington. 94(5), 2031-2035.
30. Bouer, R., Barthe, L., Philibert, C., Tournaire, C., Woodley, J., Houin, G. (1999) The roles of P-glycoprotein and intracellular metabolism in the intestinal absorption of methadone: in vitro studies using the rat everted intestinal sac. *Fundamental and Clinical Pharmacology*. 13, 494-500.
31. Geyer, J., Doring, B., Godoy, J. R., Moritz, A., Petzinger, E. (2005) Development of a PCR-based diagnostic test detecting a nt230(del4) MDR1 mutation in dogs: verification in a moxidectin-sensitive Australian Shepherd. *Journal of Veterinary Pharmacology and Therapeutics*. 28, 95-99.
32. Pulliam, J. D., Seward, R. L., Henry, R. T., Steinberg, S. A. (1985) Investigating ivermectin toxicity in Collies. *Veterinary Medicine*. 80, 33-40.
33. Lankas, G. R., Cartwright, M. E., Umbenhauer, D. (1997) P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicology and Applied Pharmacology*. 143(2), 357-365.
34. Loscher, W., Potschka, H. (2005) Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Progress in Neurology*. 76, 22-76.

35. Wilk, J. N., Bilsborough, J., Viney, J. L. (2005) The *mdr1a*<sup>-/-</sup> mouse model of spontaneous colitis: a relevant and appropriate animal model to study inflammatory bowel disease. *Immunol. Res.* 31(2), 151-160.
36. Mickisch, G. H., Pastan, I., Gottesman, M. M. (1991) Multidrug resistant transgenic mice as a novel pharmacologic tool. *Bioessays.* 13(8), 381-387.
37. Ho, G. T., Moodie, F. M., Satsangi, J. (2003) Multidrug resistance 1 gene (P-glycoprotein 170): an important determinant in gastrointestinal disease? *Gut.* 52, 759-766.
38. Natalini, C. C., Linardi, R. L. (2005) Partial Horse MDR 1 gene sequence - GenBank AY968084 *Equus caballus* multi-drug resistance p-glycoprotein 1 (MDR1) mRNA, partial cds gi|62287713|gb|AY968084.1|62287713]. Submitted (18-MAR-2005) *National Center for Biotechnology Information (NCBI), PubMed.* Accession number AY968084.
39. Leutenegger, C. M., von Rechenberg, B., Huder, J. B., Zlinsky, K., Mislin, C., Akens, M. K., Auer, J., Lutz, H. (1999) Quantitative Real-Time PCR for equine cytokine mRNA in nondecalcified bone tissue embedded in methyl methacrylate. *Calcif Tissue Int.* 65, 378-383.
40. Allen, C. A., Payne, S. L., Harville, M., Cohen, N., Russell, K. E. (2007) Validation of quantitative polymerase chain reaction assays for measuring cytokine expression in equine macrophages. *Journal of Immunological Methods.* 328, 59-69.
41. Linardi, R. L., Stokes, A. M., Barker, S. A., Short, C., Hosgood, G., Natalini, C. C. (2009) Pharmacokinetics of the injectable formulation of methadone hydrochloride administered orally in horses. *J. Vet. Pharmacol. Therap.* 32, 492-497.
42. Mouly, S., Paine, M. F. (2003) P-glycoprotein increases from proximal to distal regions of human small intestine. *Pharmaceutical Research.* 20, 10, 1595-1599.
43. Chianale, J., Vollrath, V., Wielandt, A. M., Miranda, S., Gonzales, R. *et al.* (1995) Differences between nuclear run-off and mRNA levels for multidrug resistance gene expression in the chephalocaudal axis of the mouse intestine. *Biochim. Biophys Acta.* 1264, 369-376.
44. Stephens, R. H., O'Neill, C. A., Warhurst, A., Carlson, G. L., Rowland, M., Warhurst G. (2001) Kinetic profiling of P-glycoprotein-mediated drug efflux in rat and human epithelia. *Journal of Pharmacol. Exp. Ther.* 296, 584-591.
45. Tyden, E., Tallkvist, J., Tjalve, H., Larsson, P. (2008) P-glycoprotein in intestines, liver, kidney and lymphocytes in horse. *J. Vet. Pharmacol. Therap.*, 32, 167-176.

**CHAPTER 5.**  
**THE EFFECT OF P-GLYCOPROTEIN ON METHADONE HYDROCHLORIDE FLUX**  
**IN EQUINE INTESTINAL MUCOSA**

## 5.1 Introduction

Methadone is an effective analgesic opioid used to treat moderate to severe acute and chronic pain in humans and may have a place for treatment of pain in horses. This pharmaceutical agent has unique physicochemical characteristics such as low molecular weight (below 500 Da), no hydrogen-bond donors, single oxygen and nitrogen molecules, and octanol/water partition coefficient (log P) less than 5, which are favorable for absorption and bioavailability<sup>1-5</sup>. However, variable absorption and bioavailability after oral administration of methadone have been described in people due in part, to the expression and activity of the intestinal transporter protein P-glycoprotein (P-gp). This membrane protein is believed to be a major barrier to drug absorption and bioavailability<sup>3,6,7</sup>. In horses, a previous *in vivo* study demonstrated that methadone was poorly absorbed through intestinal mucosa after oral administration (unpublished data). The poor bioavailability of methadone after oral administration may be related to the expression of P-gp in small intestinal mucosa. The effect of P-gp on methadone absorption following oral administration has not been characterized in horses.

P-glycoprotein is a large membrane protein encoded by the multidrug resistance (MDR1) gene which belongs to the ABC superfamily (subfamily B) of transporter proteins. Constitutively expressed and widely distributed in tissues, this protein alters the pharmacokinetics of pharmacologic agents by enhancing or facilitating hepatic and/or intestinal metabolism and increasing elimination<sup>8-12</sup>. However, due to its high expression in the intestinal mucosa, P-gp is a primary barrier to absorption in small intestine, which limits bioavailability<sup>6,13-15</sup>. Located in the apical membrane of enterocytes, P-gp is an energy (ATP)-dependent efflux pump that actively secretes many drugs out of the intestinal cells, back into the lumen<sup>9,12,14-18</sup>. The P-gp protein-

mediated efflux activity affects net drug transport across small intestinal mucosa and has been shown to decrease oral bioavailability of opioids<sup>6,18-22</sup>.

*In vitro* and *in situ* systems can be used to determine permeability of drugs in several species. Considering the difficulties of performing *in situ* techniques in horses and the absence of analogous equine cell lines to humans Caco-2 cells, the Ussing chamber technique has been extensively used to investigate mucosal permeability and activity of transporter proteins to estimate oral drug absorption percentage and/or predict *in vivo* drug performance<sup>23-27</sup>. Rhodamine (Rho) 123 is a cationic hydrophilic fluorescent dye recognized as a substrate of P-gp. This compound is commonly used to investigate the functional activity of P-gp in the intestinal mucosa. The absorptive and secretory transport of Rho 123 across intestinal epithelium occurs via transcellular passive diffusion. Concomitant administration of Rho 123 with other P-gp substrate drugs like methadone may enhance drug absorption due to a decrease of P-gp mediated drug transport by substrate competition. In addition, agents capable of blocking P-gp ATPase activity may increase drug absorption.

Verapamil, a calcium channel blocker, is the most extensively characterized inhibitor of P-gp and multidrug resistance (MDR) gene reversal agent used in clinical trials<sup>22</sup>. Verapamil, when incubated with methadone may decrease drug transport mediated by P-gp and increase drug absorption.

The goal of this study was to determine the effects of membrane P-gp on methadone hydrochloride flux in equine jejunal mucosa as an indicator of *in vivo* drug absorption. In addition, we investigated the effects of P-gp substrate Rho 123 and P-gp inhibitor verapamil on intestinal flux of methadone. We hypothesize that P-gp blocks the flux of methadone through equine jejunum. Rho 123 should compete with methadone for P-gp and further increase



methadone flux, and verapamil should enhance methadone flux across jejunal mucosa by blocking P-gp activity.

## 5.2 Materials and Methods

### 5.2.1 Animals

Five adult horses donated to LSU were humanely euthanatized and used in this study. Age, breed, sex, and reason for donation are listed in Table 5.1 Horses had no history or evidence of gastrointestinal disease and were free of medication for at least two weeks. This study was approved by the Louisiana State University Institutional Animal Care and Use Committee.

**Table 5.1 – Horse Information – Ussing Chamber Study**

Horses	Breed	Sex	Age	Reason for donation
1	QH	M	17	Navicular disease
2	QH	F	20+	Aged horse
3	TB	F	13	Donation – no specific reason
4	QH	F	20+	Aged horse
5	TB	M	5	Donation – no specific reason

Table represents the information of horses (breed –Quarter Horse [QH], Thoroughbred [TB], sex –male [M], female [F], age, and reason for donation) used in the Ussing chamber study

### 5.2.2 Drugs

Verapamil hydrochloride, Rho 123 (Sigma-Aldrich, St. Louis, MO, USA) and Methadone HCl (Methadone Hydrochloride Injection 10 mg/mL, *aaiPharma*, Wilmington, NC, USA) were diluted in Krebs-Ringer bicarbonate buffer (Ringer's) solution (133.48 g/L NaCl, 7.46 g/L KCl, 3.68 g/L CaCl<sub>2</sub>, 4.48 g/L MgCl<sub>2</sub>, 42 g/L NaHCO<sub>3</sub>, 0.15g/L NaH<sub>2</sub>PO<sub>4</sub>, 4.68 g/L Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) to a desired final concentration of 200 µM, 50 µM, and 3,000 ng/mL,

respectively. Verapamil hydrochloride is a  $\alpha$ 1-adrenoceptor antagonist, L-type calcium channel blocker with molecular weight of 461.06 g/mol, Rho 123 is a fluorescent dye of 380.82 g/mol molecular weight, and Methadone HCl is a synthetic  $\mu$ -opioid receptor agonist with molecular weight of 345.19 g/mol.

### **5.2.3 Tissue Preparation**

Segments of the jejunum tissue collected from five horses were rinsed with phosphate buffered saline (PBS) solution and placed in a dissection pan containing cold and oxygenated (95% oxygen/5% carbon dioxide) Ringer's solution for tissue processing. Mucosa and submucosa surfaces were carefully dissected from the underlying muscular layer and mounted in Ussing chambers.

### **5.2.4 Ussing Chamber Study Design**

Ringer's solution (15 mL) was added to each reservoir and the Ussing chamber system (World Precision Instruments, Inc., Sarasota, FL, USA) was calibrated before and after tissue assembly. The system was drained to exchange Ringer for drug solutions previously diluted at the desired concentration, including methadone, Rho 123 + methadone, verapamil + methadone, and Ringer (control). Drug solutions (methadone and Rho 123 + methadone) were added to the donor (mucosal or apical) side while Ringer's solution was added to the receptor (submucosal or basolateral) side in a final volume of 15 mL per reservoir to maintain hydrostatic pressure in both half of the chambers. Verapamil solution (15 mL) was added to both mucosal and submucosal sides of the reservoir and after 30 min, methadone at a concentration of 3,000 ng/mL was added only to the mucosal side. Ringer's solution (15 mL) was added to both sides of the chambers as the control. All solutions were randomly applied to the chambers and performed on two pieces of tissue from each horse (Table 5.2). D-Glucose (10 mM) was added to each reservoir to maintain

tissue viability. Solutions were continuously oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) and circulated in water-jacketed reservoirs with temperature maintained at 37°C. Short-circuit current (I<sub>sc</sub>) and transepithelial potential difference (PD) were recorded every 15 min, solution samples (300µL) were collected from the reservoirs of the mucosal and submucosal chamber sides at 30, 60, 90, and 120 min, and solution pH was measured at 30, 60, and 120 min. No solution was added to the reservoirs to do not alter the concentration of methadone in both chamber sides. After the completion of the study at 120 min, tissues were recovered from chambers for histologic and immunohistochemistry analysis.

### 5.2.5 Assessment of Tissue Viability

The I<sub>sc</sub> (µA) and PD (mV) were recorded and used to assess tissue viability during the study period. The PD was used as an indicator of mucosal integrity using Ringer's agar bridges connected to Ag-AgCl voltage electrodes; I<sub>sc</sub>, the current necessary to nullify PD, was used as an indicator of net Na transport across the intestinal membrane. If PD was measured between -1 and 1 mV, tissues were current clamped at 100 µA to record cIPD. Deterioration in either indicator indicates loss of tissue integrity and viability.

Electrical or tissue conductance (G) and transepithelial or tissue resistance (R) were calculated from the open-circuit PD and I<sub>sc</sub> based on Ohm's law ( $I = \frac{V}{R}$ ), where  $I$  is the short-circuit current,  $V$  is the potential difference across the tissue, and  $R$  is the transepithelial resistance. Electrical tissue conductance was calculated as follows<sup>28</sup>:

$$\text{Conductance (G)} = \frac{I_{sc}}{PD} \text{ or } \frac{I}{R}$$

Transepithelial tissue resistance (ohms/cm<sup>2</sup>) was calculated as follows<sup>28</sup>:

$$\text{Resistance (R)} = \frac{1}{\text{conductance}} \times 1,000$$

Table 5.2 – Ussing Chamber Drug Solutions

Chamber	Solutions			
<b>Mucosal</b> (donor/apical)	Methadone	Rho 123 + Methadone	Verapamil	Ringer
(after 30 min)			+ Methadone	
<b>Submucosal</b> (acceptor/basolateral)	Ringer	Ringer	Verapamil	Ringer

Table represents the drug solutions (methadone, Rho 123 + methadone, verapamil + methadone, Ringer) applied to the Ussing chambers. Drugs were dilute to a desired final concentration and added to each reservoir in a total volume of 15 mL. The final drug concentrations were: 3,000 ng/mL methadone, 50uM Rho 123, and 200 uM verapamil.

### 5.2.6 Methadone Sample Analysis

Aliquot solutions (0.3 mL) were taken from each reservoir of the chambers at 30 min, 1, 1.5, and 2 h. Relative quantitation of methadone concentration in both mucosal and submucosal sides was determined by an Enzyme Linked Immunosorbent Assay (ELISA), Methadone Direct ELISA Kit (Immunoanalysis Corporation, Pomona, CA, USA) following the manufacturer's instructions. Samples were run in duplicates in a 96 well plate. This assay was based upon the competitive binding of enzymes labeled and unlabelled antigen to the antibody. Briefly, 10 µL aliquots of samples were incubated with 100 µL of horseradish peroxidase enzyme in the microplate wells coated with polyclonal antibody (anti-Methadone). After washing the wells with 0.1% tween 20 phosphate buffered saline (PBS) and chromogenic substrate (100 µL) incubation, the blue is changed to yellow color by the addition of an acid stop solution. The intensity of color (absorbance) was read at a dual wavelength of 450 and 650 nm on a Synergy HT Multi-mode Microplate Reader (BioTek, Winooski, VT, USA) and analyzed (semi-quantitative) using KC4<sup>TM</sup> data analysis software. Color intensity was inversely proportional to the concentration of drug in the samples. Concentrations of samples were determined based on a relative standard

curve (a 10-fold dilution series with eight measuring points) derived from serial dilution of concentrated methadone at 300 ng/mL in Ringer's solution run with every plate.

#### 5.2.7 Flux of Methadone HCl Across Jejunal Mucosa

The flux of methadone HCl across the intestinal membrane (jejunum) and the effects of Rho 123 and verapamil were calculated as the percentage increase of methadone concentration in the submucosal side related to the mucosal side ( $P_{inc}$ ) over time after each drug administration, following the equation:

$$P_{inc} = \left(100 - \left(\frac{C_m - C_s}{C_m}\right) \times 100\right)$$

where  $C_m$  is the concentration of methadone in the mucosal, and  $C_s$  is the concentration in the submucosal side.

#### 5.2.8 Histology

Jejunum mucosa was recovered from the Ussing chambers after incubation with control and methadone solutions and fixed in zinc formalin for 24 h. Tissues were then embedded into paraffin, and stained with hematoxylin and eosin (H&E) using the routine methods performed by the Louisiana Animal Disease Diagnostic Laboratory at the School of Veterinary Medicine, Louisiana State University. Slides were evaluated to determine mucosal changes consistent with loss of integrity (blunting of villi and mucosal cell sluffing).

#### 5.2.9 Immunohistochemistry

Paraffin tissue blocks were also cut and prepared for immunohistochemistry analysis. Immunostaining was performed using an automated immunostainer (DAKO Autostainer, Carpinteria, CA, USA) following the instructions of Vectastain<sup>®</sup> Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). Briefly, Paraffin-embedded tissue sections were deparaffinized and rehydrated. Tissue sections were incubated for 10 min in 0.3% hydrogen

peroxidase ( $H_2O_2$ ) to block endogenous peroxidase activity, then incubate for 30 min in equine serum to block non-specific antibody binding, and then incubated for 30 min in the primary mouse monoclonal antibody [C494] (1:300). Mouse monoclonal [C494] to P-glycoprotein (ab3365, Abcam, Cambridge, MA, USA) is reported to detect a gene-specific, internal cellular epitope present only on the Mdr1 isoform of P-glycoprotein. Slides were rinsed in buffer and incubated for 30 min with a secondary antibody Bionylated Anti-Mouse IgG and ABC reagent tagged with horseradish peroxidase (Vectastain<sup>®</sup> Elite ABC Reagent). NovaRED (peroxidase) substrate kit was used to detected peroxidase activity and tissues were counterstained for 5 min with hematoxylin. A negative control that did not contain primary antibody was prepared for each tissue with no staining detected. Paraffin-embedded jejuna-tissue sections from healthy horses were used as a positive control.

#### **5.2.10 Solution pH and Osmolality**

To verify stock and bathing solution pH, indicator strips were used at before placing in Ussing chambers and after 30, 60, and 120 min. Osmolality of solutions (solute concentration) was measured using Vapro – Vapor Pressure Osmometer 5520 (Wescor, Logan, UT, USA) at the same times listed above.

#### **5.2.11 Statistical Analysis**

Statistical analysis was performed using SAS 9.1 (SAS Institute, Cary, NC, USA). Responsible variables from the electrical measurements (Isc, PD, G, and R) were ranked by horses across treatments and analyzed using a nonparametric analysis of variance (ANOVA). The flux of methadone across the intestinal epithelial membrane (mucosal or apical to submucosal or basolateral drug transport) represented as the percentage increase (Pinc) over time was analyzed

using one-way ANOVA and Tukey's test as *ad hoc* comparison between treatments. Significance was set at  $P < 0.05$ .

### 5.3 Results

There was no alteration in the pH of the drug solutions during the study. The pH measured 7-8 before and at 60 and 120 min during the 120-min period of the study. Osmolality was maintained approximately at  $260 \pm 7.9$  nmol/kg for all solutions; 253.5 nmol/kg for methadone, 262.0 nmol/kg for Rho 123 + methadone, 259.0 nmol/kg for verapamil + methadone, and 263.7 nmol/kg for Ringer's solution.

None of the solutions altered sodium transport or compromised mucosal integrity. Mean  $I_{sc}$ , PD, G, and R in tissues were not significantly different between treatments and over time, during the 120-min experimental period (Figure 5.1 and 5.2).

Histologic examination of intestinal tissues exposed to Ringer's control solution or methadone with and without Rho 123 or verapamil solution showed mild areas of ulceration, villus contactation, and debris, but these were not considered significant.

#### 5.3.1 Flux of Methadone HCl Across Jejunal Mucosa

The concentration of methadone in the mucosal chambers was stable over time, indicating no drug degradation in the Ringer's solution (Figure 5.3). Low concentrations of drug were measured on the submucosal side of the chambers when methadone was added alone or in combination with Rho 123 and there was no significant ( $P < 0.05$ ) increase in drug concentration over time. The flux of methadone across the jejunal membrane (percentage increase of drug concentration in the submucosal side related to the mucosal side over time) was significantly higher ( $P < 0.05$ ) when tissues were incubated with verapamil (Table 5.3 and Figure 5.4).

### 5.3.2 Immunohistochemistry Analysis

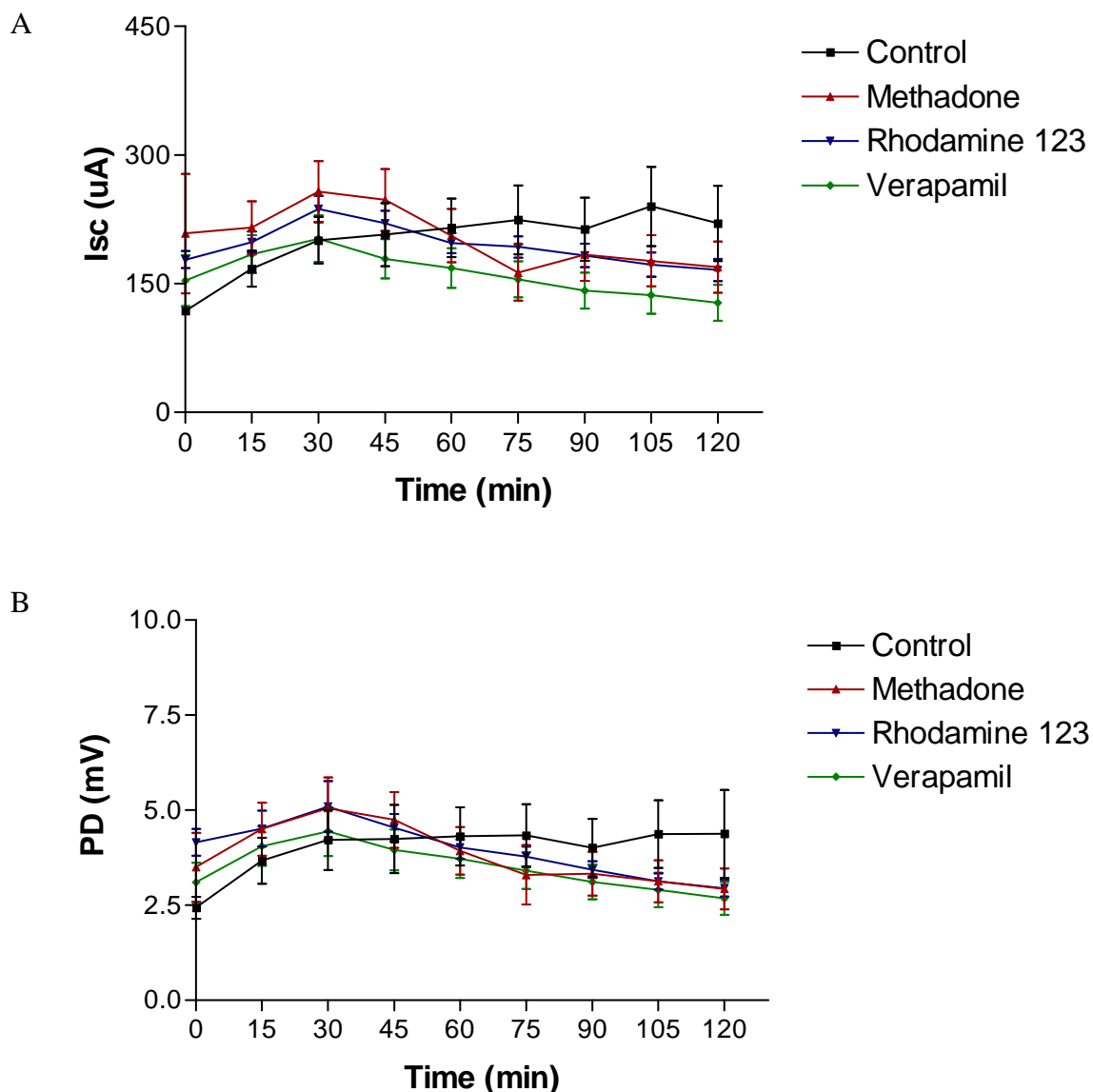
Positive immunochemical staining for P-gp was observed in the apical membrane of the jejunal epithelial cells in all horses using the monoclonal antibody C494 (Figure 5.5). There was some variability regarding the immunoreactivity (intensity of staining for P-gp) among horses (Figure 5.6). Epithelial cells (enterocytes) located in the tip of the villi in the jejunal segment demonstrated more intense staining compared to cells of the crypts (Figure 5.7). No immunoreactivity was observed in goblets cells. Staining was not observed in the negative controls (Figures 5.5 and 5.6).

### 5.4 Discussion

The Ussing chamber technique is a valid *in vitro* method to evaluate the transport or flux of drugs across intestinal mucosa and predict *in vivo* drug permeability after oral administration in horses<sup>25</sup>.

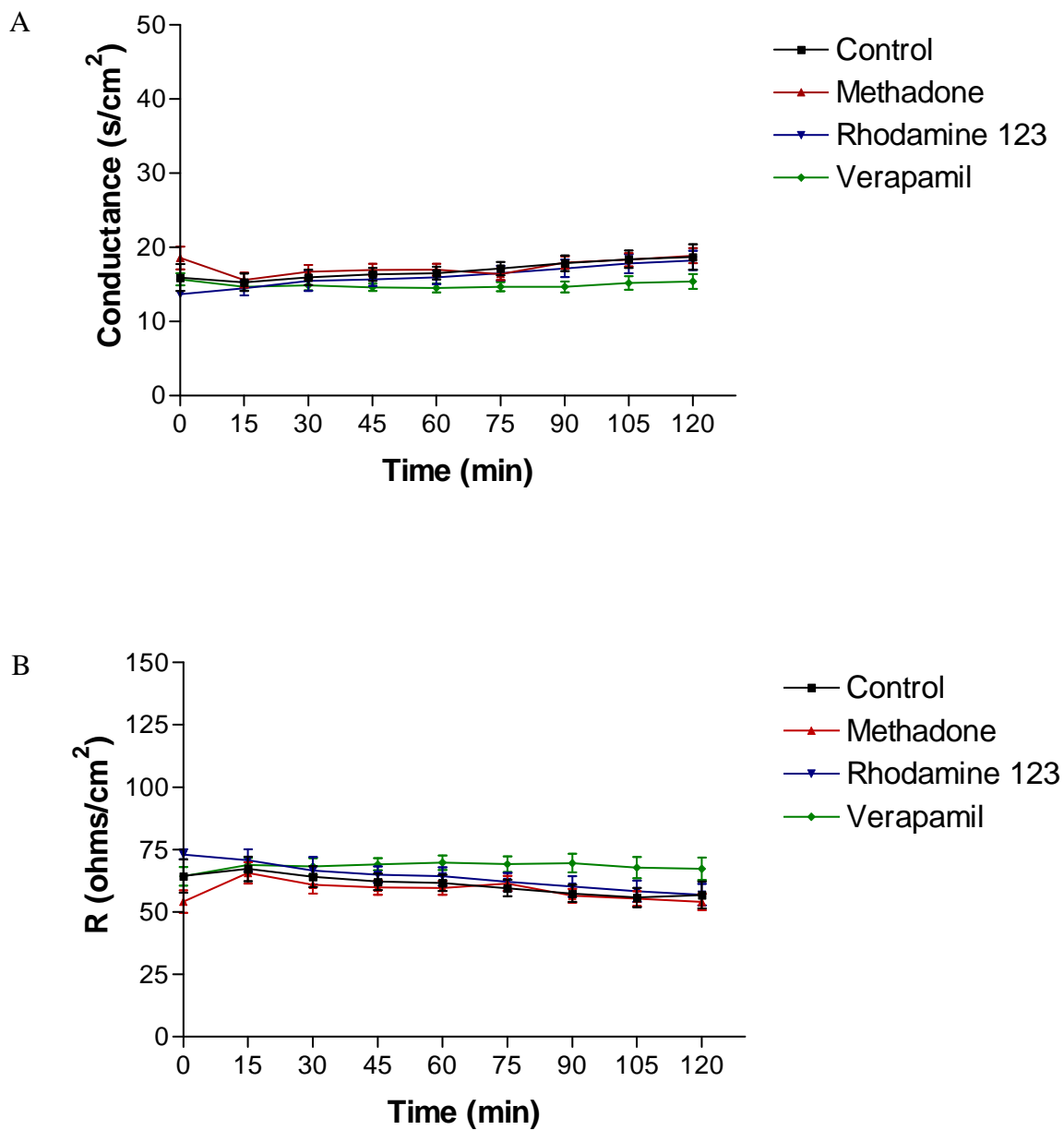
A preliminary study was performed in Ussing chambers to evaluate the effects of methadone concentration and time of exposure on tissue viability and mucosa integrity. Mucosal tissues from the jejunum collected from horses were exposed to one of the three concentrations of methadone (30, 300, and 3,000 ng/mL) or Ringer's solution (control) for 240 minutes. In that preliminary study, it was determined that mucosa exposed to methadone up to 120 min, even at the highest concentration, showed minimal mucosal pathology. After 120 min of incubation there was minimal mucosal cell sloughing and villus blunting when compared to tissues incubated for 180 min or longer. Based on these preliminary experiments, we choose an incubation period of 120 min because epithelial viability in Ussing chambers depends on the tissue, preparation, and experimental conditions<sup>27</sup>.





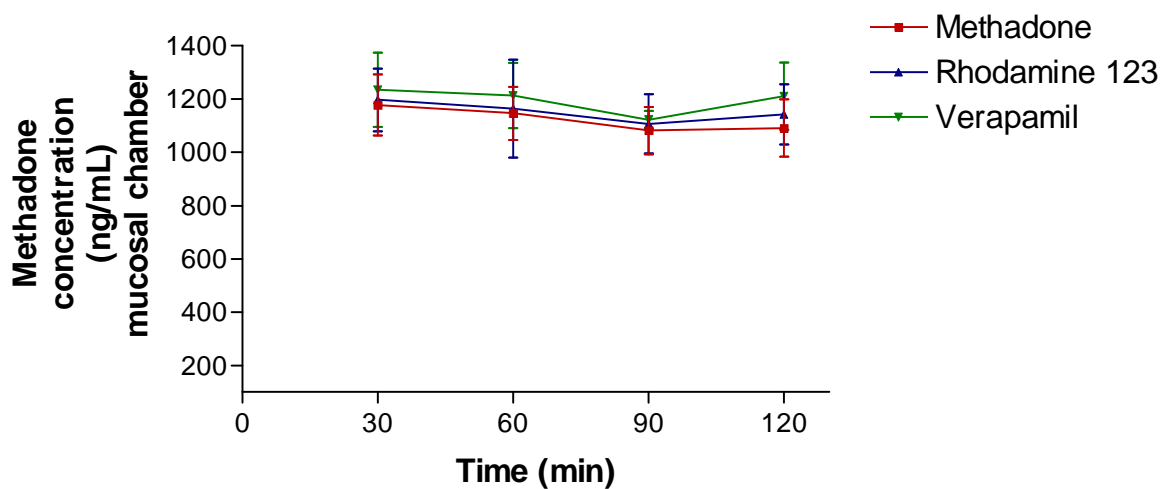
**Figure 5.1 – Short-Circuit Current (Isc) and Spontaneous Potential Difference (PD) in the Jejunum of Horses**

Means  $\pm$ SEM for Isc [ $\mu A$ ] (A) and tissue PD [mV] (B) in intestinal mucosa collected from the jejunum of five horses. Tissues were placed in Ussing chambers and the mucosal surface exposed to Ringer's solution containing methadone (at 3,000 ng/mL) with or without Rhodamine 123 (50  $\mu M$ ) or verapamil (200  $\mu M$ ). Submucosal surface was exposed to Ringer's with or without verapamil. Ringer's solution alone was used as a control. Tissues were incubated for 120 minutes.



**Figure 5.2 – Electrical Conductance (G) and Tissue Resistance (R) in the Jejunum of Horses**

Means  $\pm$ SEM for conductance [ $\text{Siemens}/\text{cm}^2$ ] (A) and tissue R [ $\text{ohms}/\text{cm}^2$ ] (B) in intestinal mucosa collected from the jejunum of five horses. Tissues were placed in Ussing chambers and the mucosal surface exposed to Ringer's solution containing methadone (at 3,000 ng/mL) with or without Rhodamine 123 (50  $\mu\text{M}$ ) or verapamil (200  $\mu\text{M}$ ). Submucosal surface was exposed to Ringer's with or without verapamil. Ringer's solution alone was used as a control. Tissues were incubated for 120 minutes.



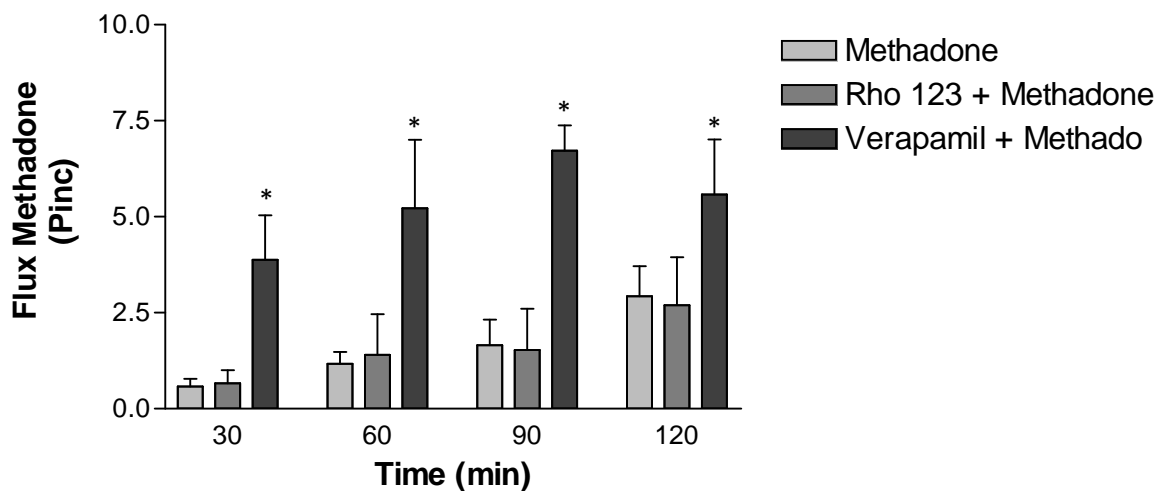
**Figure 5.3 – Concentration of Methadone in the Mucosal Side of the Ussing Chambers**

Means  $\pm$ SD of methadone concentration in the presence or absence of rhodamine 123 or verapamil measured in the mucosal side of the Ussing chambers over 120 min.

**Table 5.3 – Flux of Methadone HCl in Equine Jejunal Mucosa**

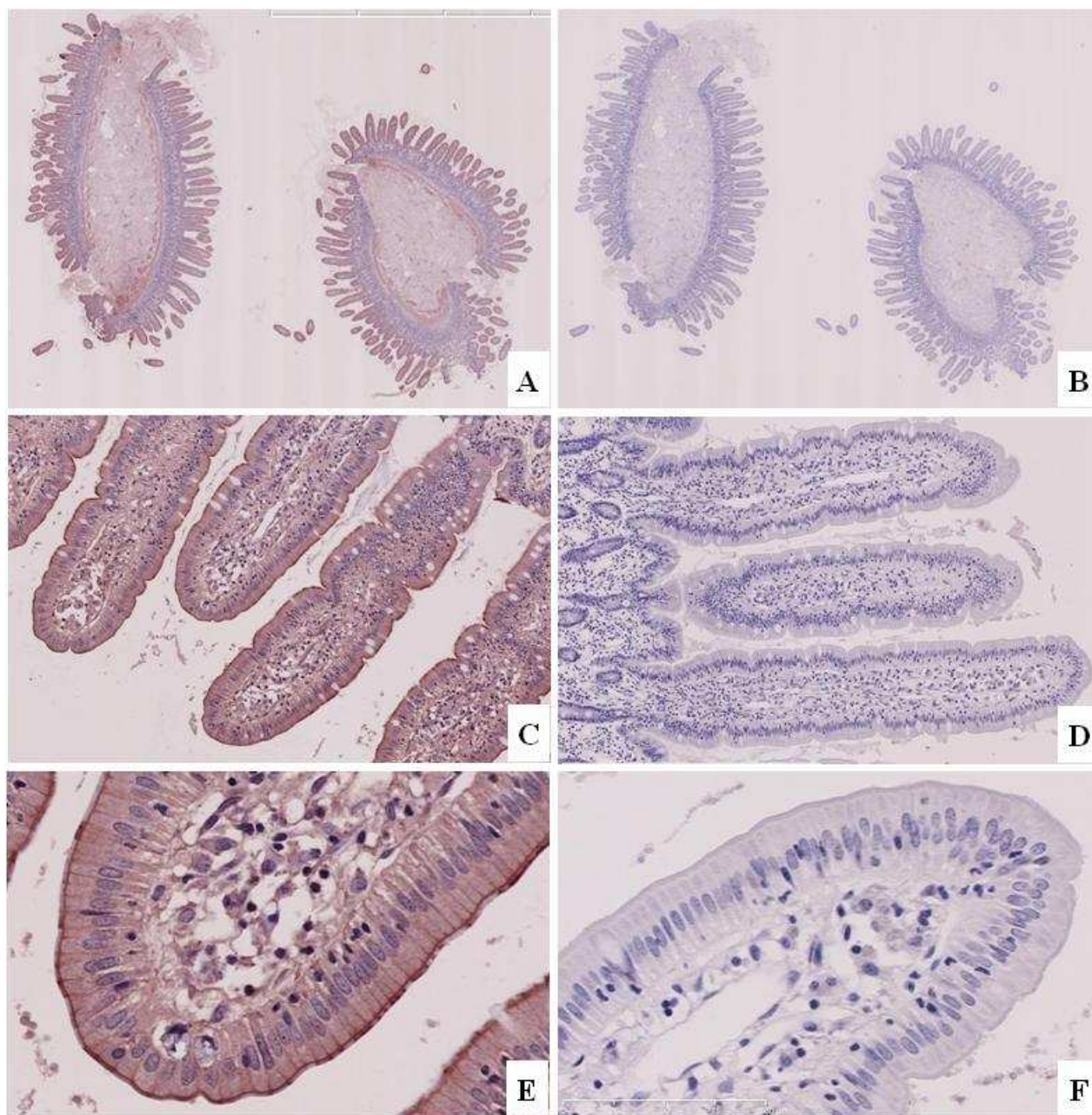
Drug Solution	Time (min)			
	30	60	90	120
Methadone	0.57 ±0.52	1.17 ±0.67	1.66 ±1.27	2.93 ±1.68
Rho 123 + Methadone	0.66 ±0.75	1.40 ±2.32	1.53 ±1.99	2.69 ±2.69
Verapamil + Methadone	3.88 * ±2.47	5.22 * ±3.31	6.72 * ±1.24	5.58 * ±3.05

Table represents the mean  $\pm$ SD percentage increase of methadone concentration in the submucosal side related to the concentration in the mucosal side of all five horses over the 120-min period. Tissues were incubated in solutions of methadone at a final concentration of 3,000 ng/mL with or without Rhodamine 123 (50  $\mu$ M) or verapamil (200  $\mu$ M). \* denotes significant differences ( $p < 0.05$ ) between drug solution at each time point.



**Figure 5.4 – Flux of Methadone HCl in Jejunal Mucosa of Horses**

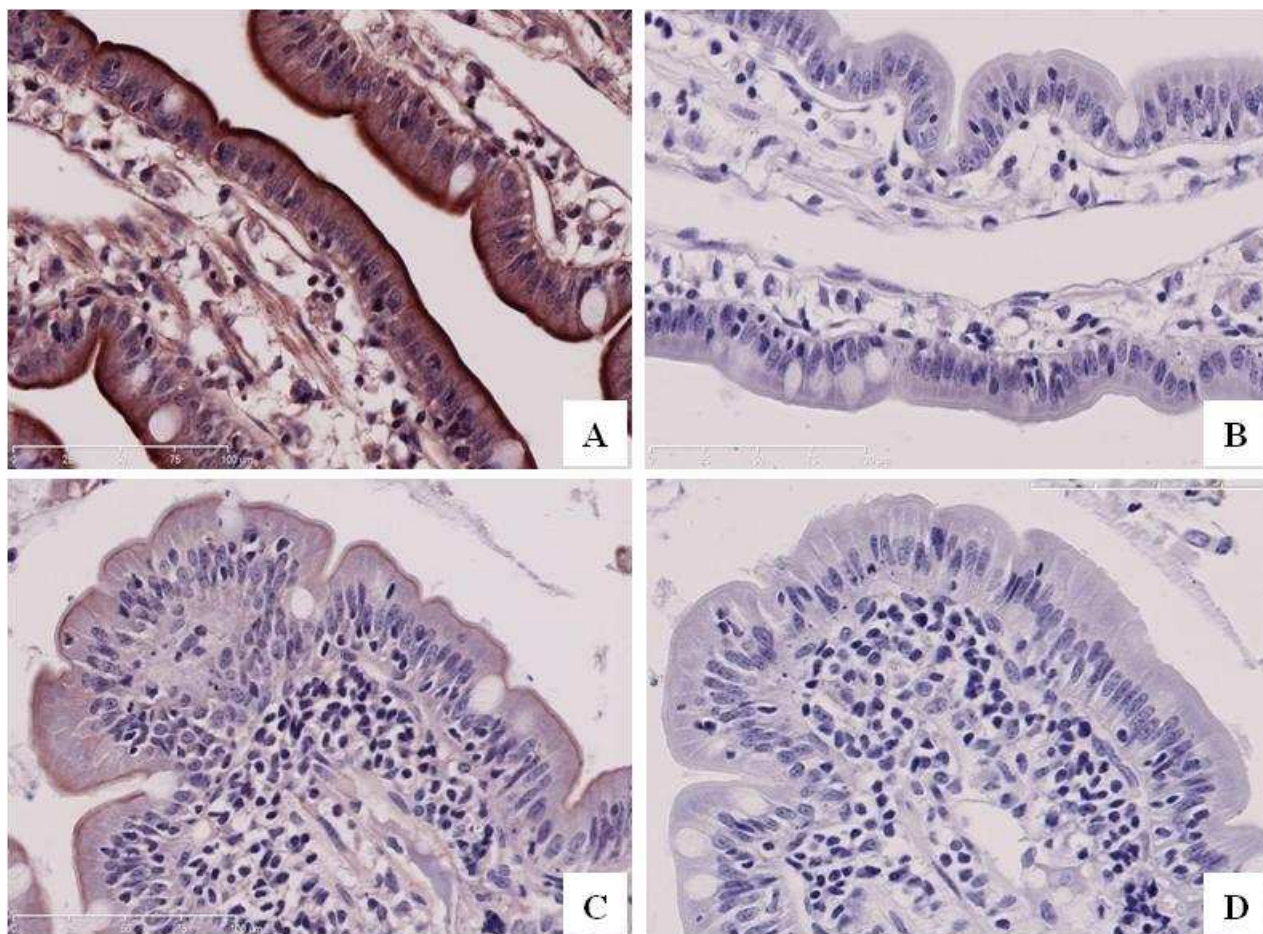
Mean  $\pm$ SD percentage increase of methadone concentration in the submucosal side related to the concentration in the mucosal side of all five horses over 120 min. Tissues were incubated in solutions of methadone at a final concentration of 3,000 ng/mL with or without Rhodamine 123 (50  $\mu$ M) or verapamil (200  $\mu$ M). \* denotes significant differences ( $p < 0.05$ ) between drug solution at each time point.



**Figure 5.5 – Immunohistochemical Staining of P-glycoprotein in the Jejunum of Horses**

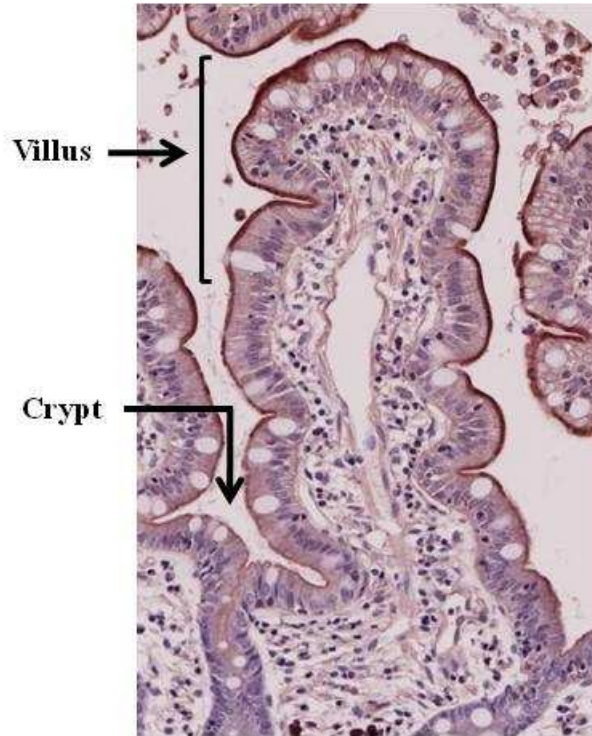
Immunohistochemical staining of P-glycoprotein with the C494 antibody (A, C, and E) and the negative control (B, D, and F) in the jejunum of a horse, observed with the source lens of 0.69X, 10X, and 40X, respectively. P-glycoprotein immunoreactivity is present in the apical membrane of intestinal epithelial cells in the villi.





**Figure 5.6 – Inter-individual Variability of Immunohistochemical Staining of P-glycoprotein in the Jejunum of Horses (40X)**

Immunohistochemical staining of P-glycoprotein with the C494 antibody in the jejunum of two different horses (A and C) and their respective negative control (B and D) observed with source lens of 40X. The intensity of P-gp immunoreactivity in the apical membrane of intestinal epithelial cells may vary among horses.



**Figure 5.7 – Immunohistochemical Staining for P-glycoprotein in the Villi of Jejunum of Horses (20X)**

Differences in the intensity of P-gp immunoreactivity were found along the villus in the jejunum of horses observed with a source lens of 20X. Epithelial cells located in the tip of the villi, where cells are more mature (enterocytes), demonstrated more intense staining compared to the poorly differentiated cells within the crypts in the jejunum of horses.



Methadone concentrations of 30 and 300 ng/mL used in the preliminary Ussing chamber study were extrapolated from results of previous *in vivo* studies performed in our laboratory. In those experiments (See Chapters 2 and 3 of this Dissertation), which investigated the oral administration (0.15 mg/kg) in horses (unpublished data)<sup>29</sup>. However, due to the limitations of the ELISA kit for detection of methadone (25 ng/mL), we were not able to detect submucosal concentrations of methadone at either of these concentrations. Thus, we choose a methadone concentration (3,000 ng/mL) in these *in vitro* experiments that we were able to detect submucosal concentrations using the ELISA kit.

In this study, jejunum tissues exposed to Ringer's solution alone and methadone (3,000 ng/mL) with or without 50  $\mu$ M Rho 123 or 200  $\mu$ M verapamil for 120 min did not alter sodium transport or compromised mucosal integrity, as indicated by no significant change in tissue Isc, PD, G, and R after 120-min exposure. Isc measures net ion transport (chloride, sodium, and potassium) across epithelium and along with PD, they are indicators of damage to the cellular Na<sup>+</sup>-K<sup>+</sup> ATPase pump and barrier disruption. No decrease in tissue R and/or increase in tissue conductance (G) confirmed that the functional integrity of the mucosa was not compromised during the period of exposure<sup>30</sup>. The osmolality and pH of all solutions were maintained at between 253.5-263.67 nmol/kg and 7 and 8, respectively, did not affect sodium transport as measured by Isc.

The flux of methadone in the equine jejunal mucosa in this experiment remained low during the 120 min incubation period. Since methadone is considered a substrate for P-gp and this protein is highly expressed in the equine intestinal mucosa, the low submucosal concentration of methadone was expected. Also, as demonstrated by the immunohistochemistry analysis, P-glycoprotein is located in the apical membrane of epithelial intestinal cells

particularly in the tip of villi of equine jejunum where cells are more mature (enterocytes) and mainly function as absorptive cells. Using the monoclonal primary antibody (C494), the expression of P-gp demonstrated to vary among horses, showing different immunostaining intensity. The C494 antibody is specific for P-gp and detects an internal epitope present only on the Mdr1 isoform of P-gp, it does not cross-react with MDR3.

After passive uptake across the intestinal epithelial cell membrane, P-gp substrate-drugs like methadone bind to P-gp (entry into the protein binding pocket) and stimulate ATPase activity, which is necessary for drug transport. Once the protein transports substrates out of the cells, conformational (allosteric) changes in the membrane domain reduce binding affinity and expose the binding site to the extracellular space to allow the release of the drug into the intestinal lumen. Subsequent to drug release, P-gp returns to its original configuration<sup>8,31-34</sup>.

P-gp-mediated drug transport can be modulated by drug-drug interaction, non-competitively binding at the substrate-binding pocket to compete with other substrates for the same binding site, or by ATP hydrolysis inhibition at the ATP binding site<sup>31</sup>. However, the incubation of tissues with methadone and Rho 123 in Ussing chambers did not increase the flux of this opioid across jejunal mucosa. Rho 123 seemed not to affect P-gp activity. Rho 123 is a well recognize substrate for P-gp, which is effluxed or transported out of the cells by this protein, but it does not work as a competitor for P-gp when administered in association with other substrates<sup>7</sup>. This could be related to the presence of multiple binding sites on the P-gp which can explain its broad substrate specificity<sup>8,12,15,35-38</sup>. However, some authors affirm that Rho123 does not bind to the P-gp; therefore, it does not act as an inhibitory agent to the drug transport mediated by this protein<sup>6</sup>. Thus, it appears that Rho 123 does not alter absorption of methadone in jejuna mucosa

In the presence of verapamil, the flux of methadone across jejunal mucosa was significantly increased, because submucosal concentrations of methadone were significantly higher after incubation. These data suggest that methadone is a substrate for P-gp in horses and this protein may play a role in this drug transport. Verapamil hydrochloride is a  $\alpha$ 1-adrenoceptor antagonist and L-type calcium channel blocker, clinically used as an anti-arrhythmic drug, and the most characterized P-gp inhibitor and multidrug resistance reversal agent. Verapamil inhibits P-gp function by blocking its ATPase activity<sup>9,22,26,31</sup>. Several uptake and transport studies using verapamil as an inhibitor and Rho123 as a substrate of P-gp demonstrated that efflux and transport across cell layers of compounds or drugs with high protein affinity are primarily affected by P-gp<sup>6,716,22,31</sup>. Despite the fact that verapamil has been demonstrated to be an *in vivo* or *in vitro* inhibitor of P-gp function, some studies suggest that this drug can also act as a protein inducer depending on the time course and concentration used. Additionally, the requirement for high doses to inhibit P-gp function or activity is cited as the major drawback for its clinical use as P-gp modulator<sup>32</sup>. It appears that verapamil functions to inhibit P-gp which increases methadone flux across equine jejunal mucosa.

In general, P-gp-mediated drug transport is described to be a saturable process (the absorptive transport mediated by P-gp decreases as concentration of the substrate increases above certain value). In Caco<sub>2</sub> cell monolayers, the transport of Rho 123 by this protein was saturable with concentrations higher than 100  $\mu$ M<sup>7</sup>. Bouer et al. (1999) demonstrated that concentration higher than 50  $\mu$ g/mL was able to saturate the P-gp-mediated transport of methadone in rats, suggesting that P-gp may become saturated with high concentrations of opioids and the efflux of this drug may reach a constant rate. This would result in a proportional increase of methadone transport across the intestinal mucosa as its concentration increases<sup>9</sup>.

However, the increase of drug concentration to inhibit protein activity and circumvent the limited intestinal absorption of drugs does not represent an advantage to the therapeutic use of opioids. It would certainly enhance the undesirable opioid-induced side effects making the use of opioids unfeasible in horses.

A wide range of natural and synthetic compounds are able to interact and modulate P-gp function. P-glycoprotein inhibitors or modulators are pharmacologically effective agents able to reverse MDR in intact cells, reducing or blocking drug efflux activity of P-gp by non-competitively binding for the same substrate-binding pocket, ATP hydrolysis inhibition, or by protein kinase C inhibition which is involved with ATP coupling to P-gp. The ideal inhibitor is a non-competitive agent that binds allosterically to the protein and is irreversible regardless substrate or inhibitor concentration. Unlike synthetic compounds such as verapamil, cyclosporine, and quinidine, which inhibitor property is dose-dependent, naturally occurring substances like coumarin and flavonoids found in plants and fruits (grapefruit, orange), capsaicin component of chilli peppers, vegetables and herbs, vitamin E and pharmaceutical excipients (tween 80, triton x-100, co-solvents) may also act as P-gp inhibitors<sup>11,12,33,35</sup>.

Like any other *in vitro* study, the Ussing chamber technique had some limitations. Due to the short period of tissue viability in the using chambers, verapamil could not be incubated longer than 30 min. For the same reason, the use of enhancers of P-gp expression was not appropriate in this study. Another limitation was the assay used to quantify the concentration of methadone in the chambers which was a semi-quantitative test. Although we found the results to be consistent, the Methadone Direct ELISA<sup>®</sup> assay relies on a change in color to measure the concentration of drug. The resulting change in color was determined in a spectrophotometer at the excitation and absorption wave length of 450 and 650 nm, respectively, based on a standard

curve with serial dilutions of a known concentration. Although ELISA offers advantages as small sample size, fast results, and cost-effective, it has limitations in detection of low concentrations of methadone (25 µg/mL).

However, the Ussing chamber was an effective technique to study the effect of P-gp on methadone HCl flux in the intestinal (jejunal) mucosa of horses as an indicator of *in vivo* drug absorption. The results obtained in this study confirmed the high expression of P-gp in the apical membrane of epithelial cells in the villi of the jejunum in horse, thus we accept our hypothesis that P-gp blocks the intestinal flux of methadone in horses. It was demonstrated that verapamil was able to decrease P-gp-mediated methadone transport across jejunal mucosa. These findings could explain at least in part, the limitation of intestinal absorption of methadone after oral administration in horses.

This study was important to evaluate the expression, localization, and activity of P-gp in the equine jejunal mucosa, but in order to fully understand the *in vivo* absorption of methadone, further investigations are needed. In addition to P-gp, there are other protein transporters such as multidrug resistance-associated proteins (MRP) that could function as efflux pumps in the intestine and contribute to the limited drug absorption as well.

## 5.5 References

1. Curatolo, W. (1998) Physical chemical properties of oral drug candidates in the discovery and exploratory development settings. *PSTT*. 1(9), 387-393.
2. Payte, J. T., Smith, J., Woods, J. (2001) Basic Pharmacology: How methadone works? The pharmacology of opioids. *National Alliance of Methadone Advocates, Educational Series*, number 5.2.
3. Eap, C. B., Buclin, T., Baumann, P. (2002) Interindividual Variability of the Clinical Pharmacokinetics of Methadone. *Clinical Pharmacokinetics*. 41(14), 1153-1193.
4. Inturrisi, C. E. (2005) Pharmacology of methadone and its isomers. *Minerva Anesthesiology*. 71, 435-437.

5. Lugo, R. A., Satterfield, K. L., Kern, S. E. (2005) Pharmacokinetics of methadone. *Journal of Pain & Palliative Care Pharmacotherapy*. 19, 13–24.
6. Dey, S., Patel, J., Anand, B. S., Jain-Vakkalagadda, B., Kaliki, P., Pal, D., Ganapathy, V., Mitra, A. K. (2003) Molecular evidence and functional expression of P-glycoprotein (MDR1) in human and rabbit cornea and cornea epithelial cell lines. *Investigative Ophthalmology & Visual Science*. 44(7), 2909-2918.
7. Troutman, M. D., Thakker, D. R. (2003) Rhodamine 123 requires carrier-mediated influx for its activity as a P-glycoprotein substrate in Caco-2 cells. *Pharmaceutical Research*. 20(8), 1192-1199.
8. Fardel, O., Lecureur, V., Guillouzo, A. (1996) The P-glycoprotein multidrug transporter. *Gen. Pharmac.*, 27(8), 1283-1291.
9. Bouer, R., Barthe, L., Philibert, C., Tournaire, C., Woodley, J., Houin, G. (1999) The roles of P-glycoprotein and intracellular metabolism in the intestinal absorption of methadone: in vitro studies using the rat everted intestinal sac. *Fundamental and Clinical Pharmacology*. 13(4), 494-500.
10. Sun, J., He, Z.G., Chen, G., Wang, S. J., Hao, X. H., Zou, M., J. (2004) Multidrug resistance P-glycoprotein: crucial significance in drug disposition and interaction. *Medical Science Monitor*. 10(1), RA5-14.
11. Martinez, M., Modric, S., Sharkey, M. et al. (2008) The pharmacogenomics of P-glycoprotein and its role in veterinary medicine. *Journal of Veterinary Pharmacology and Therapeutics*. 31, 285-300.
12. Zhou, S.F. (2008) Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*. 38(7-8), 802-832.
13. Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A. H., Smit, J. W., Meijer, D. K. F., Borst, P., Nooijen, W. J., Beijnen, J. H., van Tellingen, O. (1997) Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proceedings of the National Academy of Sciences of the United States of America*. 94(5), 2031-2035.
14. Katsura, T., Inui, K-i. (2003) Intestinal absorption of drugs mediated by drug transporters: Mechanisms and Regulation. *Drug Metab. Pharmacokin*. 18(1), 1-15.
15. Takano, M., Yumoto, R., Murakami, T. (2006) Expression and function of efflux drug transporters in the intestine. *Pharmacology and Therapeutics*. 109, 137-161.
16. Yumoto, R., Murakami, T., Nakamoto, Y., Hasegawa, R., Nagai, J., Takano, M. (1999) Transport of Rhodamine 123, a P-glycoprotein substrate across rat intestine and Caco-2 cell monolayers in the presence of cytochrome P-450 3A-related compounds. *Pharmacology and Experimental Therapeutics*. 289, 149-155.

17. Cao, X., Yu, L.X., Barbaciru, C., Landowski, C.P., Shin, H-C., Gibbs, S., Miller, H.A., Amidon, G.L., Sun, D. (2005) Permeability Dominates in Vivo Intestinal Absorption of P-gp Substrate with High Solubility and High Permeability. *Molecular Pharmacology*. 2(4), 329-340.
18. Lennernas, H. (2007) Animal data: the contributions of the Ussing chamber and perfusion system to predicting human oral drug delivery in vivo. *Advanced Drug Delivered Reviews*. 59, 1103-1120.
19. Lotsch, J., Sharke, C., Tegeder, I., Geisslinger, G. (2002) Drug Interactions with patient-controlled analgesia. *Clinical Pharmacokinetics*. 41(1), 31-57.
20. Kharasch, E. D., Hoffer, C., Whittington, D., Scheffels, P. (2003) Role of P-glycoprotein in the intestinal absorption and clinical effects of morphine. *Clinical Pharmacology and Therapeutics*. 74, 543-554.
21. Wang, J.S., Yiang, R., Taylor, R.M. et al. (2004) Brain penetration of methadone (R)- and(S)-enantiomers is greatly increased by P-gp deficiency in the blood-brain barrier of Abcb1a gene knockout mice. *Psychopharmacologia*. 173(1), 132-8.
22. Bansal, T., Misha, G., Jaggi, M., Khar, R. K., Talegaonkar, S. (2009) Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats. *European Journal of Pharmaceutical Sciences*.36, 580-590.
23. Shono, Y., Nishihara, H., Matsuda, Y., Furukawa, S., Okada, N., Fujita, T., Yamamoto, A. (2004) Modulation of intestinal P-glycoprotein function by cremophor EL and other surfactants by an in vitro diffusion chamber method using the isolated intestinal membranes. *Journal of Pharmaceutical Sciences*. 93(4), 877-885.
24. Gotoh, Y., Kamada, N., Momose, D. (2005) The advantages of the using chamber in drug absorption studies. *Journal of Biomolecular Screening*. 10(5), 2005.
25. Davis, J. L., Little, D., Blikslager, A. T., Papich, M. G. (2006) Mucosal permeability of water-soluble drugs in the equine jejunum: a preliminary investigation. *Journal of Veterinary Pharmacology and Therapeutics*. 29, 379-385.
26. Iida, A., Tomita, M., Idota, Y., Takizawa, Y., Hayashi, M. (2006) Improvement of intestinal absorption of P-glycoprotein substrate by D-tartaric acid. *Drug Metab. Pharmacokinet*. 21(5), 424-428.
27. Brown, D.R., O'Grady, S.M. (2008) The Ussing chamber and measurement of drug actions on mucosal ion transport. *Current Protocols in Pharmacology*. Unit 7.12.1 Supplement.
28. Nadeu, J.A., Andrews, F.A., Ppton, C.S., Argenzio, R.A. et al. (2003) Effects of hydrochloric, acetic, butyric, and propionic acids on pathogenesis of ulcers in the nonglandular portion of the stomach of horses. *American Journal Veterinary Research*. 64(4), 404-412.

29. Linardi, R.L., Stokes, A.M., Barker, S.A., Short, C., Hosgood, G., Natalini, C.C. (2009). Pharmacokinetics of the injectable formulation of methadone hydrochloride administered orally in horses. *J. vet. Pharmacol. Therap*, **32**, 492-497.
30. Andrews, F.A., Buchanan, B.R., Smith, S.H., Elliot, S.B., Saxton, A.M. (2006) In vitro effects of hydrochloric acid and various concentrations of acetic, propionic, butyric, or valeric acids on bioelectric properties of equine gastric squamous mucosa. *American Journal Veterinary Research*. 67(11), 1873-1882.
31. Hassan, H. E., Myers, A. L., Coop, A., Eddington, N. D. (2009) Differential involvement of P-gp (ABCB1) in permeability, tissue distribution, and antinociceptive active of methadone. Buprenorphine, and diprenorphine: *In vitro* and *in vivo* Evaluation. *Journal of Pharmaceutical Sciences*. 98(12), 4928-4940.
32. Matheny, C.J., Lamb, M.W., Brouwer, K.L.R., Pollack, G.M. (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy*, 21(7), 778-796.
33. Sharom, F.J. (2006) Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABCB1). *Biochemistry and Cell Biology*. 84, 979-992.
34. Crettol, S., Digon, P., Golay, K. P., Brawand, M., Eap, C. B. (2007) In vitro P-glycoprotein-mediated transport of (R)-, (S)-, (R,S)-methadone, LAAM and their main metabolites. *Pharmacology*. 80, 304-311.
35. Fromm, M.F. (2004) Importance of P-glycoprotein at blood-tissue barriers. *TRENDS in Pharmacological Sciences*. 25(8), 423-429.
36. Mealey, K.L. (2004) Therapeutic implications of the MDR-1 gene. *Journal of Veterinary Pharmacology and Therapeutics*. 27, 257-264.
37. Cascorbi, I. (2006) Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. *Pharmacology & Therapeutics*, 112, 547-473.
38. Kimura, Y., Morita, S., Matsuo, M., Ueda, K. (2007) Mechanism of multidrug recognition by MDR1/ABCB1. *Cancer Science*. 98(9), 1303-1310.



**CHAPTER 6.**  
**CONCLUSIONS AND FUTURE DIRECTIONS**

These studies were significant to demonstrate that oral administration of methadone is not associated with undesirable effects and it may result in drug serum or plasma concentration high enough to induce analgesia in horses. In addition, *in vitro* studies explored the pharmacogenomics in the equine species and investigated whether the transporter protein P-glycoprotein (P-gp) plays a role in the absorption and pharmacokinetics of methadone after oral administration to horses.

Through *in vivo* studies, the oral pharmacokinetics of methadone was characterized as dose-independent and short-half life in horses. Oral administration of methadone at a single dose up to 0.4 mg/kg demonstrated to be safe, viable, and not associated with induced side effects in horses. Drug concentrations measured in horse serum were at least as high as the effective or therapeutic concentration range for methadone established in humans. As reported in other species, inter-variability regarding the pharmacokinetic parameters was also observed in horses.

Highly lipophilic drugs like methadone usually diffuse across the small intestinal epithelium preferably via a passive transcellular process. This process occurs mainly in the jejunum due to the presence of villi and microvilli which increase the absorptive surface area. However, the absorption of methadone in our study appeared to be limited in the small intestine of horses. This finding was supported by significant low area under the drug concentration-time curve and low bioavailability after intragastric drug administration. Furthermore, *in vivo* studies suggested the oral mucosa play an important role in the absorption of methadone after oral administration to horses. Methadone is an opioid drug that encloses specific physicochemical characteristics related to high drug solubility and high permeability which determine a successful drug molecule consistent with good absorption and great bioavailability but, the mechanisms of this opioid uptake following oral administration remain to be fully characterized in horses.

In many species, P-gp is considered one of the major intestinal barriers with critical impact on oral absorption, bioavailability, and disposition of numerous drugs and substrates for this protein. Methadone is one of the opioid agents recognized as P-gp substrate. Due to the lack of information in horses, *in vitro* studies addressed the partial sequencing of the equine multidrug resistance (MDR1) gene and determined its expression levels in several tissues such as oral mucosa, sublingual, esophagus, stomach (glandular and nonglandular parts), small intestine (duodenum, jejunum, ileum), and liver. The expression of MDR1 gene was significantly higher in the small intestine with highest expression on the jejunum and ileum segments. In the jejunum of horses, P-gp was recognized by specific antibodies and located in the apical membrane of epithelial cells (enterocytes) in the villi.

As an indicator of *in vivo* absorption of methadone through the small intestine of horses after its oral administration, the Ussing chamber technique was used to study *in vitro*, the flux of methadone in the intestinal mucosa. Equine jejunal mucosa were exposed to solutions containing methadone at final concentration of 3,000 ng/mL with or without Rhodamine [Rho] 123 (50  $\mu$ M) or verapamil (200  $\mu$ M) over a 120-min period. Our studies suggested that P-gp may play a role in the intestinal transport of methadone, limiting its flux across jejunal mucosa. To determine the flux of drug (percentage increase of drug in the submucosal side), aliquots were collected over time, from both mucosal and serosal sides of the chambers and concentration were measured using a relative quantitation method. Percentage increase was determined by the difference in concentration between submucosal and mucosal samples related to the total concentration in the mucosal side.

The P-gp blocked the flux of methadone across jejunal mucosa from the apical to the submucosal side. The Rho 123, a P-gp substrate, did not alter the P-gp-mediated drug transport

and did not increase the intestinal flux of methadone. Methadone flux in equine jejunal mucosa was significantly higher in the presence of the P-gp inhibitor verapamil, a calcium channel blocker. Our studies suggested that methadone is a P-gp substrate and this protein is able to alter drug permeability and decreases *in vivo* drug absorption.

Further studies are still necessary to expand the knowledge about oral pharmacokinetics of opioids and to determine the pharmacodynamics of methadone and its therapeutic range in horses. Other *in vitro* approaches would contribute to investigate the role of P-gp on the limited intestinal opioid absorption. In addition, *in vivo* protein-modulation studies would help to determine in which extent the P-gp accounts for the limited absorption of methadone and to investigate the possible involvement of other transporter proteins. Circumventing the limited absorption of methadone mediated by P-gp, complementary studies could improve oral bioavailability of methadone and other opioids in horses.

## BIBLIOGRAPHY

Abbott, F. S., Slatter, J. G., Burton, R., Kang, G. I. (1985) Methadone metabolism in the rat in vivo: identification of a novel formamide metabolite. *Xenobiotica*. 15(2), 129-140.

Alexandrova, R. (1998) Multidrug resistance and P-glycoprotein. *Experimental Pathology and Parasitology*. 62-65.

Allen, C. A., Payne, S. L. Harville, M., Cohen, N., Russell, K. E. (2007) Validation of quantitative polymerase chain reaction assays for measuring cytokine expression in equine macrophages. *Journal of Immunological Methods*. 328, 59-69.

Andrews, F.A., Buchanan, B.R., Smith, S.H., Elliot, S.B., Saxton, A.M. (2006) In vitro effects of hydrochloric acid and various concentrations of acetic, propionic, butyric, or valeric acids on bioelectric properties of equine gastric squamous mucosa. *American Journal Veterinary Research*. 67(11), 1873-1882.

Bansal, T., Misha, G., Jaggi, M., Khar, R. K., Talegaonkar, S. (2009) Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats. *European Journal of Pharmaceutical Sciences*. 36, 580-590.

Batheja, P., Thakur, R., Michniak, B. (2007) Basic biopharmaceutics of buccal and sublingual absorptions, chapter 9, 175-202. In: Touitou, E., Barry, B.W. *Enhancement in drug delivery*. CRC Press, Taylor & Francis Group, LLC, USA. p.615.

Birkett, D. J. (2002) *Pharmacokinetics made easy – Revised ed.* McGraw-Hill, Sydney, NY. p.132.

Bouer, R., Barthe, L., Philibert, C., Tournaire, C., Woodley, J., Houin, G. (1999) The roles of P-glycoprotein and intracellular metabolism in the intestinal absorption of methadone: in vitro studies using the rat everted intestinal sac. *Fundamental and Clinical Pharmacology*. 13(4), 494-500.

Boulton, D. M., Arnaud, P., Devane, C. L. (2001) Pharmacokinetics and pharmacodynamics of methadone enantiomers after a single oral dose of racemate. *Clinical Pharmacology & Therapeutics*. 70(1), 48-57.

Bowsher, D. (2003) The treatment of neuropathic pain. IN: Bountra C., Munglani R., Schmidt W.K. *Pain*. 549-558.

Brady, J. M., Cherrington, N. J., Hartley, D. P., Buist, S. C., Li N., Klaassen, C. D. (2002) Tissue distribution and chemical induction of multiple drug resistance genes in rats. *Drug Metabolism and Disposition*. 30(7), 838-844.

Branson K. R., Gross M. E., Booth N. H. (1995) Opioid agonists and antagonists. IN: Adams, H. R. *Veterinary Pharmacology and Therapeutics*. 7ed. 274-310.

- Brown, D. R., O'Grady, S. M. (2008) The Ussing chamber and measurement of drug actions on mucosal ion transport. *Current Protocols in Pharmacology*. Unit 7.12.1-7.12.17 Supplement.
- Cao, X., Yu, L.X., Barbaciru, C., Landowski, C.P., Shin, H-C., Gibbs, S., Miller, H.A., Amidon, G.L., Sun, D. (2005) Permeability Dominates in Vivo Intestinal Absorption of P-gp Substrate with High Solubility and High Permeability. *Molecular Pharmacology*. 2(4), 329-340.
- Cascorbi, I. (2006) Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. *Pharmacology & Therapeutics*, 112, 547-473.
- Centeno, C., Vara, F. (2005) Intermittent subcutaneous methadone administration in the management of cancer pain. *Journal of Pain & Palliative Care Pharmacotherapy*. 19(2), 7-12.
- Chan, L-N. (2008) Opioid analgesics and the gastrointestinal tract. *Practical Gastroenterology. Nutrition Issues in Gastroenterology*. Series 64-65, 37-50.
- Chhabra, S., Bull, J. (2008) Methadone. *American Journal of Hospice and Palliative Medicine*. 25(2), 146-150.
- Chianale, J., Vollrath, V., Wielandt, A. M., Miranda, S., Gonzales, R. *et al.* (1995) Differences between nuclear run-off and mRNA levels for multidrug resistance gene expression in the chephalocaudal axis of the mouse intestine. *Biochim. Biophys Acta*. 1264, 369-376.
- Clark, D. C. (2008) Understanding methadone metabolism. *Anesthesiology*. 108, 351-352.
- Clark, J. O., Clark, T. P. (1999) Analgesia. *Clinical Pharmacology and Therapeutics. Veterinary Clinics of North America: Equine Practice*. 15(3), 705-723.
- Clarke D. M. (2006) Structure and function of membrane transport proteins. <http://biochemistry.utoronto.ca/clarke/bch.html>. Accessed on Dec. 2009.
- Clarke, K. W.; Paton, B. S. (1988) Combined use of detomidine with opiates in the horse. *Equine Veterinary Journal*. 20(1), 331-334.
- Cohen, M. J., Schecter, W. P. (2005) Perioperative pain control: A strategy for management. *Surgical Clinics of America*. 85, 1243-1257.
- Collaghan, R., Riordan, J. R. (1993) Synthetic and natural opiates interact with P-glycoprotein in multidrug-resistant cells. *The Journal Biological Chemistry*. 268(21), 16050-16064.
- Combie, J., Dougherty, J., Nugent, C. E., Tobin, T. (1979) The pharmacology of narcotic analgesics in the horse. IV. Dose and time response relationships for behavioral responses to morphine, meperidine, pentazocine, anileridine, methadone, and hydromorphone. *J. Equine Med Surg*. 3, 377-385.

Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Briedler, J. L., Melamed, M. R., Bertino, J. R. (1989) Multidrug resistance gene is expressed by endothelial cells at blood-brain barrier sites. *Proceedings Nat. Acad. Sci. of USA*. 86, 695-698.

Crettol, S., Digon, P., Golay, K. P., Brawand, M., Eap, C. B. (2007) In vitro P-glycoprotein-mediated transport of (R)-, (S)-, (R,S)-methadone, LAAM and their main metabolites. *Pharmacology*. 80, 304-311.

Cummins, C. L., Salphati, L., Reid M. J., Benet, L. Z. (2003) In vivo modulation of intestinal CYP3A metabolism by P-glycoprotein: Studies using the rat single-pass intestinal perfusion model. *Journal of Pharmacology and Experimental Therapeutics*. 305(1), 306-314.

Curatolo, W. (1998) Physical chemical properties of oral drug candidates in the discovery and exploratory development settings. *PSTT*. 1(9), 387-393.

Dagenais, C., Graff, C. L., Pollack, G. M. (2004) Variable modulation of opioid brain uptake by P-glycoprotein in mice. *Biochemical Pharmacology*. 67, 269-276.

Dale, O., Hoffer, C., Sheffels, P., Kharasch, E. D. (2002) Disposition of nasal, intravenous, and oral methadone in healthy volunteers. *Clinical Pharmacology & Therapeutics*. 72, 536-545.

Dale, O., Sheffels, P., Kharasch, E. D. (2004) Bioavailabilities of rectal and oral methadone in healthy subjects. *Br J Clin Pharmacol*. 58 (2), 156-162.

Davis, J. L., Papich, M. G., Weingarten, A. (2006) The pharmacokinetics of orbifloxacin in the horse following oral and intravenous administration. *J. vet. Pharmacol. Therap*. 29, 191-197.

Davis, J. L., Little, D., Blikslager, A. T., Papich, M. G. (2006) Mucosal permeability of water-soluble drugs in the equine jejunum: a preliminary investigation. *Journal of Veterinary Pharmacology and Therapeutics*. 29, 379-385.

Deshpande, M. A., Holden, R. R., Gilron, I. (2006) The impact of therapy on quality of life and mood in neuropathic pain: What is the effect of Pain reduction? *Anesthesia and Analgesia*. 102, 1473-1479.

Dey, S., Patel, J., Anand, B. S., Jain-Vakkalagadda, B., Kaliki, P., Pal, D., Ganapathy, V., Mitra, A. K. (2003) Molecular evidence and functional expression of P-glycoprotein (MDR1) in human and rabbit cornea and cornea epithelial cell lines. *Investigative Ophthalmology & Visual Science*. 44(7), 2909-2918.

Dhawan, B. N., Cesselin, F., Raghubir, R., Reisine, T., Bradley, P. B., Portoghese, P. S., Hamon, M. (1996) International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacological Reviews*. 48(4), 567-592.

- Di Pietro, A., Dayan, G., Conseil, G. et. al. (1999) P-glycoprotein-mediated resistance to chemotherapy in cancer cells: using recombinant cytosolic domains to establish structure-function relationships. *Brazilian Journal of Medical and Biological Research*. 32, 925-939.
- Driessen, B. (2008) Perioperative pain management in the horse: Can we effectively inhibit/prevent 'wind-up'? *The 14<sup>th</sup> Annual International Veterinary Emergency and Critical Care Symposium (IVECCS)*. Phoenix, AZ. September 17-21, 2008.
- Eap, C. B., Buclin, T., Baumann, P. (2002) Individual variability of the clinical pharmacokinetics of methadone. Implications for the treatment of opioid dependence. *Clinical Pharmacokinetics*. 41(14), 1153–1193.
- Evans, G. S., Flint, N., Somers, A. S., Eyden, B., Potten, C. S. (1992) The development of a method for the preparation of rat intestinal epithelial cell primary cultures. *Journal of Cell Science*. 101, 219-231.
- Fardel, O., Lecureur, V., Guillouzo, A. (1996) The P-glycoprotein multidrug transporter. *Gen. Pharmac.*, 27(8), 1283-1291.
- Fakhoury, M., Litalien, C., Medard, Y., Cavé, H., Ezzahir, N., Peuchmaur, M., Jacqz-Aigrain E. (2005) Localization and mRNA expression of CYP3A and P-glycoprotein in human duodenum as a function of age. *Drug Metab. Dispos.* 33, 1603-1607.
- Foster, D. J., Somogyi, A. A., Bochner, F. (1999) Methadone N-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4. *British Journal of Clinical Pharmacology*. 47, 403-412.
- Freshney, R. I. (2005) *Culture of Animal Cells: A Manual of Basic Technique*. Fifth Edition. John Wiley & Sons, Hoboken, NJ, USA. p.642.
- Fromm, M. F. (2004) Importance of P-glycoprotein at blood-tissue barriers. *TRENDS in Pharmacological Sciences*. 25(8), 423-429.
- Geyer, J., Doring, B., Godoy, J. R., Moritz, A., Petzinger, E. (2005) Development of a PCR-based diagnostic test detecting a nt230(del4) MDR1 mutation in dogs: verification in a moxidectin-sensitive Australian Shepherd. *Journal of Veterinary Pharmacology and Therapeutics*. 28, 95-99.
- Gibaldi M., Prescott L. (1983) *Handbook of Clinical Pharmacokinetics*. Australia: ADIS Health Science Press, Australia, p.1208.
- Glowaski, M. M. (2007) Treating animal pain: The clinical perspective. *Veterinary Pharmacology 2007. American Academy of Veterinary Pharmacology and Therapeutics. 15<sup>th</sup> Biennial Symposium* Monterey, CA. May 20-24, 2007.



Golaz, J. L., Vonlaufen, N., Hemphill, A., Burgener, I. A. (2007) Establishment and characterization of a primary canine duodenal epithelial cell culture. *In Vitro Cell. Dev. Biol.-Animal*. 43, 176-185.

Gotoh, Y., Kamada, N., Momose, D. (2005) The advantages of Ussing chamber in drug absorption studies. *Journal of Biomolecular Screening*. 10(5), 517-523.

Gottesman, M. M., Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* 62, 385-427.

Gourlay, G. K., Wilson, P. R., Glynn, C. J. (1982) Pharmacodynamics and pharmacokinetics of methadone during the perioperative period. *Anesthesiology*. 57, 458-467.

Gourlay, G.K., Cherry, D.A., Cousins, M.J. (1986). A comparative study of the efficacy and pharmacokinetics of oral methadone and morphine in the treatment of severe pain in patients with cancer. *Pain*. 25(3), 297-312.

Gourlay, G. K., Willis, R. J., Lamberty, J. (1986) A double-blind comparison of the efficacy of methadone and morphine in postoperative pain control. *Anesthesiology*. 64, 322-327.

Gourlay, G. K., Willis, R. J., Wilson, P. R. (1984) Postoperative pain control with methadone: Influence of supplementary methadone doses and blood concentration-response relationships. *Anesthesiology*. 61, 19-26.

Gourlay, G. K. (2005) Advances in opioid pharmacology. *Support Care Cancer*. 13, 153-159.

Hagedorn, H. W., Meiser, H., Zankl, H., Schulz, R. (1999) Methadone screening of racehorses. *Journal of Analytical Toxicology*. 23, 609-614.

Hall, L. W., Clarke, K. W., Trim, C. M. (2001) *Veterinary Anaesthesia* 10ed. W.B.Saunders. p.561.

Hardman, J. G., Limbird, L. E. (2001) Goodman & Gilman's The Pharmacological Basis of Therapeutics. United States: McGraw-Hill, US, p. 2148.

Hassan, H. E., Myers, A. L., Coop, A., Eddington, N. D. (2009) Differential involvement of P-gp (ABCB1) in permeability, tissue distribution, and antinociceptive active of methadone, buprenorphine, and diprenorphine: *In vitro* and *in vivo* Evaluation. *Journal of Pharmaceutical Sciences*. 98(12), 4928-4940.

Hellyer, P. W., Bai, L., Supon, J.; Quail, C., Wagner, A. E., Mama, K. R., Magnusson, K. R. (2003) Comparison of opioid and alpha-2 adrenergic receptor binding in horse and dog brain using radioligand autoradiography. *Veterinary Anaesthesia and Analgesia*. 30, 172-182.

Hewitt, D. J. (2000) The use of the NMDA-receptor antagonists in the treatment of chronic pain. *The Clinical Journal of Pain*. 16, S73-S79.

- Ho, G. T., Moodie, F. M., Satsangi, J. (2003) Multidrug resistance 1 gene (P-glycoprotein 170): an important determinant in gastrointestinal disease? *Gut*. 52, 759-766.
- Ieiri, I., Takane, H., Otsubo, K. (2004) The *MDR1* (ABCB1) gene polymorphism and its clinical implications. 49(9), 553-576.
- Iida, A., Tomita, M., Idota, Y., Takizawa, Y., Hayashi, M. (2006) Improvement of intestinal absorption of P-glycoprotein substrate by D-tartaric acid. *Drug Metab. Pharmacokinet.* 21(5), 424-428.
- Inturrisi, C. E. (2005) Pharmacology of methadone and its isomers. *Minerva Anesthesiology*. 71, 435-437.
- Juliano, R. L., Ling, V. (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta*. Amsterdam. 455, 152-162.
- Kaeffer, B. (2002) Mammalian intestinal epithelial cells in primary culture: A mini-review. *In Vitro Cell. Dev. Biol.-Animal*. 38, 123-134.
- Kamerling, S. (1988) Dose related effects of the kappa agonist U-50, 488H on behavior, nociception and autonomic response in the horse. *Equine Veterinary Journal*. 20(2), 114-118.
- Katsura, T., Inui, K-I. (2003) Intestinal absorption of drugs mediated by drug transporters: Mechanisms and Regulation. *Drug Metab. Pharmacokin.* 18(1), 1-15.
- Kharasch, E. D., Hoffer, C., Whittington, D., Scheffels, P. (2003) Role of P-glycoprotein in the intestinal absorption and clinical effects of morphine. *Clinical Pharmacology and Therapeutics*. 74, 543-554.
- Kharasch, E. D., Hoffer, C., Whittington, D. (2004) The effect of quinidine, used as a probe for the involvement of p-glycoprotein, on the intestinal absorption and pharmacodynamics of methadone. *British Journal of Clinical Pharmacology*. 57, 600-610.
- Kharasch, E. D., Hoffer, C., Whittington, D., Walker A., Bedynek, P. S. (2009) Methadone pharmacokinetics are independent of cytochrome P4503A (CYP3A) activity and gastrointestinal drug transport: insights from methadone interactions with ritonavir/indinavir. *Anesthesiology*. 110, 660-672.
- Kimura, Y., Morita, S., Matsuo, M., Ueda, K. (2007) Mechanism of multidrug recognition by MDR1/ABCB1. *Cancer Science*. 98(9), 1303-1310.
- Kukanich, B., Borum, S. L. (2008) The disposition and behavioral effects of methadone in Greyhounds. *Veterinary Anaesthesia and Analgesia*. 35, 242-248.
- Kukanich, B., Lascelles, B. D., Aman, A. M., Mealey, K. L., Papich, M. G. (2005) The effects of inhibiting cytochrome P450 3A, p-glycoprotein, and gastric acid secretion on the oral

bioavailability of methadone in dogs. *Journal of Veterinary Pharmacology and Therapeutics*. 28, 461-466.

Lankas, G. R., Cartwright, M. E., Umbenhauer, D. (1997) P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicology and Applied Pharmacology*. 143(2), 357-365.

Lauretti, G. R. (2006) Highlights in opioid agonists and antagonists. *Expert Rev. Neurotherapeutics*. 6(4), 613-622.

Lennernas, H. (2007) Animal data: The contributions of the Ussing chamber and perfusion systems to predicting human oral drug delivery *in vivo*. *Advanced Drug Delivery Reviews*. 59, 1103-1120.

Leutenegger, C. M., von Rechenberg, B., Huder, J. B., Zlinsky, K., Mislin, C., Akens, M. K., Auer, J., Lutz, H. (1999) Quantitative Real-Time PCR for equine cytokine mRNA in nondecalfied bone tissue embedded in methyl methacrylate. *Calcif Tissue Int*. 65, 378-383.

Li, Q., Sai, Y., Kato, Y., Tamai, I., Tsuji, A. (2003) Influence of drugs and nutrients on transporter gene expression levels in Caco-2 and LS180 intestinal epithelial cell lines. *Pharmaceutical Research*. 20(8), 1119-1124.

Linardi, R. L., Stokes, A. M., Barker, S. A., Short, C., Hosgood, G., Natalini, C. C. (2009). Pharmacokinetics of the injectable formulation of methadone hydrochloride administered orally in horses. *J. vet. Pharmacol. Therap*. 32, 492-497.

Loscher, W., Potschka, H. (2005) Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Progress in Neurobiology*. 76(1), 22-76.

Lotsch, J., Sharke, C., Tegeder, I., Geisslinger, G. (2002) Drug Interactions with patient-controlled analgesia. *Clinical Pharmacokinetics*. 41(1), 31-57.

Lotsch, J., Geisslinger, G. (2006) Current evidence for a genetic modulation of the response to analgesics. *Pain*. 121, 1-5.

Lugo, R. A., Satterfield, K. L., Kern, S. E. (2005) Pharmacokinetics of methadone. *Journal of Pain & Palliative Care Pharmacotherapy*. 19, 13-24.

Macartney, K. K., Baumgart, D. C., Carding, S. R., Brubaker, J. O., Offit, P. A. (2000) Primary murine small intestinal epithelial cells, maintained in long-term culture, are susceptible to rotavirus infection. *Journal of Virology*. 74(12), 5597-5603.

Maier, A., Zimmermann, C., Beglinger, C., Drewe, J., Gutmann, H. (2007) Effects of budesonide on P-glycoprotein expression in intestinal cell lines. *British Journal of Pharmacology*. 150, 361-368.

- Marchetti, S., Mazzant, R., Beijnen, J. H., Schellens, J. H. M. (2007) Clinical relevance of drug-drug and herb-drug interactions mediated by the ABC Transporter ABCB1 (MDR1, P-glycoprotein). *The Oncologist*. 12, 927-941.
- Martinez, M., Modric, S., Sharkey, M. et al. (2008) The pharmacogenomics of P-glycoprotein and its role in veterinary medicine. *Journal of Veterinary Pharmacology and Therapeutics*. 31, 285-300.
- Matheny, C. J., Lamb, M. W., Brouwer, K. L. R., Pollack, G. M. (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy*. 21(7), 778-796.
- Maxwell, L. K., Thomasy, S. M., Slovis, N., Kollias-Baker, C. (2003) Pharmacokinetics of fentanyl following intravenous and transdermal administration in horses. *Equine Veterinary Journal*. 35(5), 484-490.
- Mealey, K. L., Bentjen, S. A., Gay, J. M., Cantor, G. H. (2001) Ivermectin sensitivity in collies is associated with a deletion mutation of the *mdr1* gene. *Pharmacogenetics*. 11(8), 727-733.
- Mealey, K. L., Munyard, K. A., Bentjen, S. A. (2005) Frequency of the mutant MDR1 allele associated with multidrug sensitivity in a sample of herding breed dogs living in Australia. *Veterinary Parasitology*. 131(3-4), 193-196.
- Mealey, K. L. (2004) Therapeutic implications of the MDR-1 gene. *Journal of Veterinary Pharmacology and Therapeutics*. 27, 257-264.
- Mickisch, G. H., Pastan, I., Gottesman, M. M. (1991) Multidrug resistant transgenic mice as a novel pharmacologic tool. *Bioessays*. 13(8), 381-387.
- Miller, D. S., Bauer, B., Hartz, A. M. S. (2008) Modulation of P-glycoprotein at the blood-brain barrier: Opportunities to improve central nervous system pharmacotherapy. *Pharmacological Reviews*. 60(2), 196-209.
- Mizuno, N., Niwa, T., Yotsumoto, Y., Sugiyama, Y. (2003) Impact of drug transporter studies on drug discovery and development. *Pharmacological Reviews*. 55(3), 425-461.
- Molina, P. E. (2005) Opioids and opiates: analgesia with cardiovascular, haemodynamic and immune implications in critical illness. *Journal of Internal Medicine*. 259, 138-154.
- Mouly, S., Paine, M. F. (2003) P-glycoprotein increases from proximal to distal regions of human small intestine. *Pharmaceutical Research*. 20, 10, 1595-1599.
- Muir, W. W., Skarda, R. T., Sheehan, W. C. (1978) Cardiopulmonary effects of narcotic agonists and a partial agonist in horses. *Am. J. Vet. Res.* 39(10), 1632-1635.

Mukhopadhyay, T., Batsakis, J. G., Kuo, M. T. (1988) Expression of the mdr (P-glycoprotein) gene in Chinese hamster digestive tracts. *Journal of the National Cancer Institute*. 80(4), 269-275.

Nadeu, J.A., Andrews, F.A., Ppton, C.S., Argenzio, R.A. et al. (2003) Effects of hydrochloric, acetic, butyric, and propionic acids on pathogenesis of ulcers in the nonglandular portion of the stomach of horses. *American Journal Veterinary Research*. 64(4), 404-412.

Natalini, C. C., Linardi, R. L. (2005) Partial Horse MDR 1 gene sequence - GenBank AY968084 *Equus caballus* multi-drug resistance p-glycoprotein 1 (MDR1) mRNA, partial cds gi|62287713|gb|AY968084.1|[62287713]. Submitted (18-MAR-2005) *National Center for Biotechnology Information (NCBI), PubMed*. Accession number AY968084.

Natalini, C. C., Linardi, R. L. (2006) Analgesic effects of epidural administration of hydromorphone in horses. *American Journal of Veterinary Research*. 67(1), 11–15.

Natalini, C. C., Robinson, E. P. (2000) Evaluation of the analgesic effects of epidurally administered morphine, alfentanil, butorphanol, tramadol, and U50488H in horses. *American Journal of Veterinary Research*. 61(12), 1579-1586.

Natalini, C. C., Robinson, E. P. (2003) Effects of epidural opioid analgesics on heart rate, arterial blood pressure, respiratory rate, body temperature, and behavior in horses. *Vet.Therapeutics*. 4(4), 364-375.

Natalini, C. C., Polydoro, A. S., Linardi, R. L. (2006) Analgesic effects of subarachnoidally administered hyperbaric opioids in horses. *American Journal of Veterinary Research*. 67(6), 941-946.

Neuhoff, S. (2005) Refine *in vitro* models for prediction of intestinal drug transport. Role of pH and extracellular additives in the Caco-2 cell model. Dissertation. *Acta Universitatis Upsaliensis*. p.84.

Oda, Y., Kharasch, E. D. (2001) Metabolism of methadone and levo-alpha-acethylmetadol (LAAM) by human intestinal cytochrome P450 3A4 (CYP3A4), potential contribution of intestinal metabolism to presystemic clearance and bioactivation. *The Journal of Pharmacology and Experimental Therapeutics*. 298, 1021-1032.

Olbrich, V. H, Mosing, M. (2003) A comparison of the analgesic effects of caudal epidural methadone and lidocaine in the horse. *Vet. Anaesthesia and Analgesia*. 30(3):156-164.

Olsen, L. (2007) Drugs in horses: Pharmacokinetics and Pharmacodynamics. *Doctoral Thesis. Swedish University of Agricultural Sciences*. p.56.

Page, G. G. (2005) Immunologic effects of opioids in the presence of absence of pain. *Journal of Pain and Symptom Management*. 29(5), 25-29.

- Payte, J. T., Smith, J., Woods, J. (2001) Basic Pharmacology: How methadone works? The pharmacology of opioids. *National Alliance of Methadone Advocates, Educational Series*, number 5.2.
- Pippi, N. L., Lumb, W. V. (1979) Objective tests of analgesic drugs in ponies. *Am. J. Vet. Res.* 40(7), 1082-1086.
- Pippi, N. L., Lumb, W. V., Fialho, S. A. G., Scott, R. J. (1979) A model of evaluation pain in ponies. *J. Eq. Med. Surg.* 3, 430-435.
- Pulliam, J. D., Seward, R. L., Henry, R. T., Steinberg, S. A. (1985) Investigating ivermectin toxicity in Collies. *Veterinary Medicine.* 80(6), 33-40.
- Robertson, S. A., Taylor, P. M. (2004) Pain management in cats -past, present, and future. Part 2. Treatment of pain -clinical pharmacology. *Journal of Feline Medicine and Surgery.* 6, 321-333.
- Robinson, E. P., Natalini, C. C. (2002) Epidural anesthesia and analgesia in horses. *The Veterinary Clinics of North America: Equine practice.* 18(1), 61-82.
- Rowlingson J. C., Murphy T. M. (2000) Chronic pain. IN: Miller R.D. *Anesthesia* 5ed. 2351-2379.
- Sadeque A. J. M., Wandel C., He, H. et al. (2000) Increased drug delivery to the brain by glycoprotein inhibition. *Clinical Pharmacology and Therapeutics.* 68(3), 231-237.
- Schinckel, A. H., Smit, J. J., Telling, O., Beijnen, J. H., Wagenaar, E. et al. (1994) Disruption of the Mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell.* 77, 491-502.
- Schinkel A. H, Wagenaar E., Mol C. A. A. M., Deemter L. V. (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation.* 97, 2517-2524.
- Schrickx, J., Fink-Gremmels, J. (2007) P-glycoprotein-mediated transport of oxytetracycline in the Caco-2 cell model. *J. Vet. Pharmacol. Therap.* 30, 25-31.
- Shargel, L., Wu-Pong, S., Yu, A. B. C. (2005) *Applied Biopharmaceutics & Pharmacokinetics*, 5th edn. Mc Graw-Hill, Medical Publishing Division, White Plains p.768.
- Sharom, F. J. (2006) Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABCB1). *Biochemistry and Cell Biology.* 84, 979-992
- Shimoyama, N., Shimoyama, M., Elliot, K. J., Inturrisi, C. E. (1997) D-methadone is antinociceptive in the rat formalin test. *The Journal of Pharmacology and Experimental Therapeutics.* 283(2), 648-652.

Shir, Y., Shenkman, Z., Shavelson, V., Davidson, E., Rosen, G. (1998) Oral Methadone for the treatment of severe pain in hospitalized children: A report of five cases [Case Report]. *The Clinical Journal of Pain*. 14(4), 350-353.

Shono, Y., Nishihara, H., Matsuda, Y., Furukawa, S., Okada, N., Fujita, T., Yamamoto, A. (2004) Modulation of intestinal P-glycoprotein function by cremophor EL and other surfactants by an in vitro diffusion chamber method using the isolated intestinal membranes. *Journal of Pharmaceutical Sciences*. 93(4), 877-885.

Sinatra, R. S. (2006) Peripherally acting Mu-opioid-receptor antagonists and the connection between postoperative ileus and pain management: The anesthesiologist's view and beyond. *Journal of PeriAnesthesia Nursing*. 21(2A), S16-S23.

Skarda, R. T. (1996) Local and regional anesthetic and analgesic techniques: horses. In: Thurmon JC, Tranquilli WJ, Benson GJ, eds. *Lumb & Jones' veterinary anesthesia*. Baltimore, MD: Williams and Wilkins. p.448-478.

Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A. H., Smit, J. W., Meijer, D. K. F., Borst, P., Nooijen, W. J., Beijnen, J. H., van Tellingen, O. (1997) Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proceedings of the National Academy of Sciences of the United States of America*. 94(5), 2031-2035.

Stephens, R. H., O'Neill, C. A., Warhurst, A., Carlson, G. L., Rowland, M., Warhurst G. (2001) Kinetic profiling of P-glycoprotein-mediated drug efflux in rat and human epithelia. *Journal of Pharmacol. Exp. Ther*. 296, 584-591.

Stormer, E., von Moltke, L. L., Perloff, M. D., Greenblatt, D. J. (2001) P-glycoprotein interactions of nefazodone and trazodone in cell culture. *Journal Clin. Pharmacol*. 41, 708-714.

Sun, J., He, Z. G., Chen, G. Wang, S. J., Hao, X. H., Zou, M., J. (2004) Multidrug resistance P-glycoprotein: crucial significance in drug disposition and interaction. *Medical Science Monitor*. 10(1), RA5-14.

Takano, M., Yumoto, R., Murakami, T. (2006) Expression and function of efflux drug transporters in the intestine. *Pharmacology and Therapeutics*. 109, 137-161.

Thiebaut, F., Tsuruo, T., Gottsman, M. M., Pastan, I., Willingham, M. C. (1987) Cellular localization in normal human tissues. *Proceedings of the National Academy of Sciences of USA*. 84, 7735-7738.

Thompson, S. J., Koszdin, K., Bernards, C. B. (2000) Opioid-induced analgesia is increased and prolonged in mice lacking P-glycoprotein. *Anesthesiology*. 92(5), 1392-1399.

Thurmon, J. C., Tranquilli, W. J., Benson, G. J. (1996) *Lumb & Jones' veterinary anesthesia*. Baltimore, MD: Williams and Wilkins p.928.

Tobin, T. (1978) Narcotic analgesics and the opiate receptor in the horse. *J. Equine Med Surg.* 2, 397-399.

Tobin, T., Combie, J., Shults, T. (1979) Pharmacology review: actions of central stimulant drugs in the horse. II. *J. Equine Med Surg.* 3, 102-109.

Tobin, T., Combie, J., Shults, T., Dougherty, J. (1979) The pharmacology of narcotic analgesics in the horse. III. Characteristics of the locomotor effects of fentanyl and apomorphine. *J. Equine Med Surg.* 3, 284-288.

Tobin, T. (1981) Horses running happy: fentanyl, morphine, and the other narcotic analgesics. In *Drugs and the Performance Horse*. Ed. Tobin, T., pp. 199–215. Charles C. Thomas Publisher, Springfield.

Toombs, J. D., Kral, L. A. (2005) Methadone treatment for pain states. *American Family Physician.* 71(7), 1353-1358.

Total, R. A., Sheffels, P., Roberts, T., Whittington, D., Thummel, K., Kharasch, E. D. (2008) Role of CYP2B6 in stereoselective human methadone metabolism. *Anesthesiology.* 108(3), 363-374.

Troutman, M. D., Thakker, D. R. (2003) Rhodamine 123 requires carrier-mediated influx for its activity as a P-glycoprotein substrate in Caco-2 cells. *Pharmaceutical Research.* 20(8), 1192-1199.

Tyden, E., Tallkvist, J., Tjalve, H., Larsson, P. (2008) P-glycoprotein in intestines, liver, kidney and lymphocytes in horse. *J. Vet. Pharmacol. Therap.* 32, 167-176.

Utoguchi, N., Watanabe, Y., Suzuki, T., Maehara, J., Matsumoto, Y., Matsumoto, M. (1997) Carrier-mediated transport of monocarboxylic acids in primary cultured epithelial cells from rabbit oral mucosa. *Pharmaceutical Research.* 14(3), 320-324.

Walker, A. F. (2007) Sublingual administration of buprenorphine for long-term analgesia in the horse (Short Communication). *The Veterinary Record.* 160, 808-809.

Wang, J. S., Ruan, Y., Taylor, R. M., Donovan, J. L., Markowitz, J. S., DeVane, C. L. (2004) Brain penetration of methadone (R)-and(S)-enantiomers is greatly increased by P-gp deficiency in the blood-brain barrier of Abcb1a gene knockout mice. *Psychopharmacology.* 173(1), 132-138.

Watanabe, S., Ishikawa, Y., Hara, H., Hanzawa, K., Mukoyama, H. (1997) A method of primary cell culture for establishing equine long-term culture cell lines. *J. Equine Sci.* 8(4), 95-99.

Weinberg, D. S., Intrussi, C. E., Reidenberg, B., Moulin, D. E., Nip, T. J., Wallenstein, S., Houde, R. W., Foley, K. M. (1988) Sublingual absorption of selected opioid analgesics. *Clinical pharmacology and therapeutics.* 44(3), 335-342.



Wells, S. M., Glerum, L. E., Papich, M. G. (2008) Pharmacokinetics of butorphanol in cats after intramuscular and buccal transmucosal administration. *American Journal of Veterinary Research*. 69(12), 1548-1554.

Wikipedia contributors, "Methadone," *Wikipedia, The Free Encyclopedia*, <http://en.wikipedia.org/w/index.php?title=Methadone&oldid=329778261> (accessed December 11, 2009).

Wilk, J. N., Bilsborough, J., Viney, J. L. (2005) The *mdr1a*<sup>-/-</sup> mouse model of spontaneous colitis: a relevant and appropriate animal model to study inflammatory bowel disease. *Immunol. Res.* 31(2), 151-160.

Xie, R., Hammarlund-Udenaes, M., de Boer, A. G., de Lange, E. C. M. (1999) The role of P-glycoprotein in blood-brain barrier transport of morphine: transcortical microdialysis studies in *mdr1a* (-/-) and *mdr1b* (+/+) mice. *British Journal of Pharmacology*. 128, 563-568.

Yamaoka, K., Nakagawa, T., Uno, T. (1978) Statistical moments in pharmacokinetics 1. *Journal of Pharmacokinetics and Biopharmaceutics*. 6, 547-558.

Yoshimura, M., Furue, H. (2006) Mechanisms for the anti-nociceptive actions of the descending noradrenergic and serotonergic systems in the spinal cord. *Journal Pharmacol. Sci.* 101, 107-117.

Yumoto, R., Murakami, T., Nakamoto, Y., Hasegawa, R., Nagai, J., Takano, M. (1999) Transport of Rhodamine 123, a P-glycoprotein substrate across rat intestine and Caco-2 cell monolayers in the presence of cytochrome P-450 3A-related compounds. *Pharmacology and Experimental Therapeutics*. 289, 149-155.

Zhou, S. F. (2008) Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*. 38(7-8), 802-832.

Zimmermann, C., Gutmann, H., Hruz, P., Gutzwiller, J-P., Beglinger, C., Drewe, J. (2005) Mapping of multidrug resistance gene 1 and multidrug resistance associated protein isoform 1 to 5 mRNA expression along the human intestinal tract. *The American Society for Pharmacology and Experimental Therapeutics*. 33(2), 219-224.

**APPENDIX:  
LETTER OF PERMISSION**

JOHN WILEY AND SONS LICENSE  
TERMS AND CONDITIONS

Apr 21, 2010

This is a License Agreement between Renata L Linardi ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

**All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.**

License Number	2374970385011
License date	Feb 23, 2010
Licensed content publisher	John Wiley and Sons
Licensed content publication	Journal of Veterinary Pharmacology and Therapeutics
Licensed content title	Pharmacokinetics of the injectable formulation of methadone hydrochloride administered orally in horses
Licensed content author	LINARDI R. L., STOKES A. M., BARKER S. A., et al
Licensed content date	Jan 1, 0010
Start page	492
End page	497
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Order reference number	
Total	0.00 USD
Terms and Conditions	

**TERMS AND CONDITIONS**

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at <http://myaccount.copyright.com>).

Terms and Conditions

1. The materials you have requested permission to reproduce (the "Materials") are protected by copyright.
2. You are hereby granted a personal, non-exclusive, non-sublicensable, non-transferable, worldwide, limited license to reproduce the Materials for the purpose specified in the licensing process. This license is for a one-time use only with a maximum distribution equal to the number that you identified in the licensing process. Any form of republication granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before may be distributed thereafter). Any electronic posting of the Materials is limited to one year from the date permission is granted and is on the condition that a link is placed to the journal homepage on Wiley's online journals publication platform at [www.interscience.wiley.com](http://www.interscience.wiley.com). The Materials shall not be used in any other manner or for any other purpose. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher and on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Material. Any third party material is expressly excluded from this permission.
3. With respect to the Materials, all rights are reserved. No part of the Materials may be copied, modified, adapted, translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Materials, or any of the rights granted to you hereunder to any other person.
4. The Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc or one of its related companies (WILEY) or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.
5. WILEY DOES NOT MAKE ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING,

WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in a writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

13. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

14. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

15. This Agreement shall be governed by and construed in accordance with the laws of England and you agree to submit to the exclusive jurisdiction of the English courts.

BY CLICKING ON THE "I ACCEPT" BUTTON, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.

V1.2

**Gratis licenses (referencing \$0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.**

**If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10739940. Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.**

**Make Payment To:**  
**Copyright Clearance Center**  
**Dept 001**  
**P.O. Box 843006**  
**Boston, MA 02284-3006**

**If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2374970385011 and noting the reason for cancellation.**

**Questions? [customercare@copyright.com](mailto:customercare@copyright.com) or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.**

## **VITA**

Renata Lehn Linardi was born in February, 1976, in Americana, Sao Paulo, Brasil. She grew up in Americana, where she completed her secondary education at the Anglo Cidade de Americana in 1993. She attended the Universidade Estadual Paulista – UNESP-Jaboticabal, Sao Paulo, Brasil, earning a degree of Doctor of Veterinary Medicine in January 2001.

After graduation, Dr. Linardi was accepted into the residency program in Large Animal Surgery and Anesthesiology at the Veterinary Hospital “Governador Laudo Natel”, UNESP-Jaboticabal. In January 2003, she successfully completed her residency. As a result of her research endeavors during that time, Dr. Linardi enrolled at the same institution into the Master Program in Veterinary Surgery where she completed her M.S degree in March of 2004.

Dr. Linardi was invited to travel to the United States of America to work as a Research Assistant in the Department of Veterinary Clinical Sciences at the School of Veterinary Medicine at the Louisiana State University in August 2004. She was accepted into the Louisiana State University Graduate School in January 2006, and she is currently a candidate for the degree of Doctor of Philosophy in veterinary medical sciences which will be awarded in May 2010.