Determination of Pharmacokinetic Parameters of Subcutaneous Zyklophin by Quantitative Determination of Zyklophin in Mouse Plasma by LCMS/MS Analysis For Assessment As A Potential Therapeutic Agent In The Treatment of Cocaine Addiction

By

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Abstract

Pharmaceutical agents targeting the μ opioid receptors (MOR) have been widely used in the treatment and management of pain. However, serious side effects and the prevalence of abuse have complicated their administration. This has increased the interest of developing pharmaceutical agents that selectively bind to the κ opioid receptors (KOR). Recent investigation has determined that KOR agonists exhibit a decreased liability for addiction as well as respiratory depression in comparison to MOR agonists. Selectively binding antagonists to the KOR have only been utilized historically as pharmacological tools. Additionally, KOR antagonists show promising potential in therapeutic treatment of depression and anxiety, as well as opiate and cocaine addiction.

KOR antagonists that are metabolically stable have been shown to penetrate the CNS. However, peptide-based KOR-selective antagonists are metabolized by proteases and are expected to have a shorter activity than non-peptide KOR-selective antagonists with increased metabolic stability. Through the incorporation of unnatural amino acids as well as conformational constraints in the C terminus, zyklophin experiences increased enzymatic stability. Initial testing has shown that zyklophin crosses the blood brain barrier to antagonize the KOR in the CNS with in vivo administration and that its duration of activity after a system dose is less than 12 hours, whereas a non-peptide KOR antagonist, such as nor-BNI, exhibits duration of activity weeks after a single dose is administered. This prolonged activity complicates its use as a pharmacological tool and as a potential therapeutic agent whereby increasing the importance of peptide-based KOR antagonist.

Currently, the FDA has not approved a drug for the prevention of relapse due to cocaine addiction. Peptide-based drugs have been shown to be successful pharmaceutical agents exhibiting high activity, high specificity, low toxicity, and minimal drug-drug interaction. Due to

modifications of the C-terminus, zyklophin has an increased metabolic stability. Thus, zyklophin is a good lead compound for the treatment of cocaine addiction and relapse.

This thesis is dedicated to my wife, Melissa Mann,
for always pushing me to do my absolute best at everything I have ever attempted and for her continual encouragement.

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I will forever be grateful to my wife for her support throughout this challenging process. Melissa, without you this road would have been so much more difficult. Thank-you for always being understanding over the past five years. Thank-you for knowing when to

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Sola scriptura, Sola fide, Sola gratia, Solus christus, Soli Deo gloria

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Chapter One:

Cocaine Addiction and Potential Treatment by the *κ* Opioid Receptor Antagonist Zyklophin

1.1. Cocaine

Cocaine is an illicit, highly addictive narcotic that directly affects the brain by interfering with the dopamine transport system. It has had a long history of abuse in America, and over the past decade cocaine has become an increasing prominent part of the European drug scene. In Europe, cocaine is the most abused illicit stimulant in the southern and western countries and the second most abused illicit stimulant in the northern and eastern countries. [1]. Cocaine is derived from coca leaves and is sold in two forms: a water-soluble hydrochloride salt and a water-insoluble freebase. The water soluble hydrochloride salt can be injected intravenously and both forms can be snorted or smoked all of which allows for rapid to immediate absorption into the body.

Cocaine affects the neural system in the ventral tegmental area (VTA) of the brain. Nerve fibers exist in the VTA that extend into areas of the brain involved in reward. When cocaine passes through the blood brain barrier and enters the VTA, dopamine levels rise resulting in increased neural activity. In normal brain function, a neuron releases dopamine into a synapse where it binds to dopamine receptors on a neighboring neuron sending a signal to that neuron relating to reward, reinforcement, motivation, arousal, cognitive control, or motor control.

Following binding, dopamine is then removed from the synapse and recycled by the dopamine transporter. The transporter binds the neurotransmitter and pumps it out of the synaptic cleft back into the presynaptic neuron, where it is taken up into storage vesicles. Cocaine binds tightly at the dopamine transporter and forms a complex that blocks the transporter's function [2]. The dopamine transporter can no longer perform its reuptake function, and thus dopamine accumulates in the synaptic cleft. See Figure 1.1 for a diagram illustrating the mechanism of action for cocaine.

Cocaine binds in a manner that directly stabilizes the dopamine transporter in the open outward-facing conformation, whereas other stimulants stabilize in the closed conformation [2]. Cocaine binds in such a way as to inhibit a hydrogen bond innate to the dopamine transporter's function by attaching to the active site of the protein. The hydrogen bond will not form since it is then blocked from formation due to the tightly locked orientation of the cocaine molecule. The affinity for the transporter is not what is involved in so much as the conformation and binding properties to where and how on the transporter the cocaine binds [2].

As a result of repeated exposure, cocaine abuse leads to the reward pathway becoming less sensitive to positive reinforcement or pleasurable feelings of natural rewards (i.e. food, water, exercise, sex) as well as to cocaine itself. As a result, high doses of cocaine, as well as more frequent applications, are required to achieve a euphoric sensation. These increased doses and more frequent abuses often occur in binge behavior. Due to the binge behavior, users can and usually experience one or more of the following: irritability, restlessness, panic attacks, paranoia, or psychosis [3].

The National Survey on Drug Use and Health estimates that in 2008 there were 1.9 million current cocaine abusers and of which 359,000 were abusing crack cocaine. In 2008, 1.4 million Americans met the *Diagnostic and Statistical Manual of Mental Disorders* criteria for dependence or abuse of cocaine in the past 12 months [3]. A Drug Abuse Warning Network report showed that in 2008 cocaine was involved in 482,188 of nearly 2 million emergency visits due to drug abuse [4]. In 2014 a report showed that the mortality rate of cocaine users is over five times greater than for the general population [5]. Additionally, another 2014 report showed that after cannabis, cocaine is the most frequently used illicit drug globally [6].

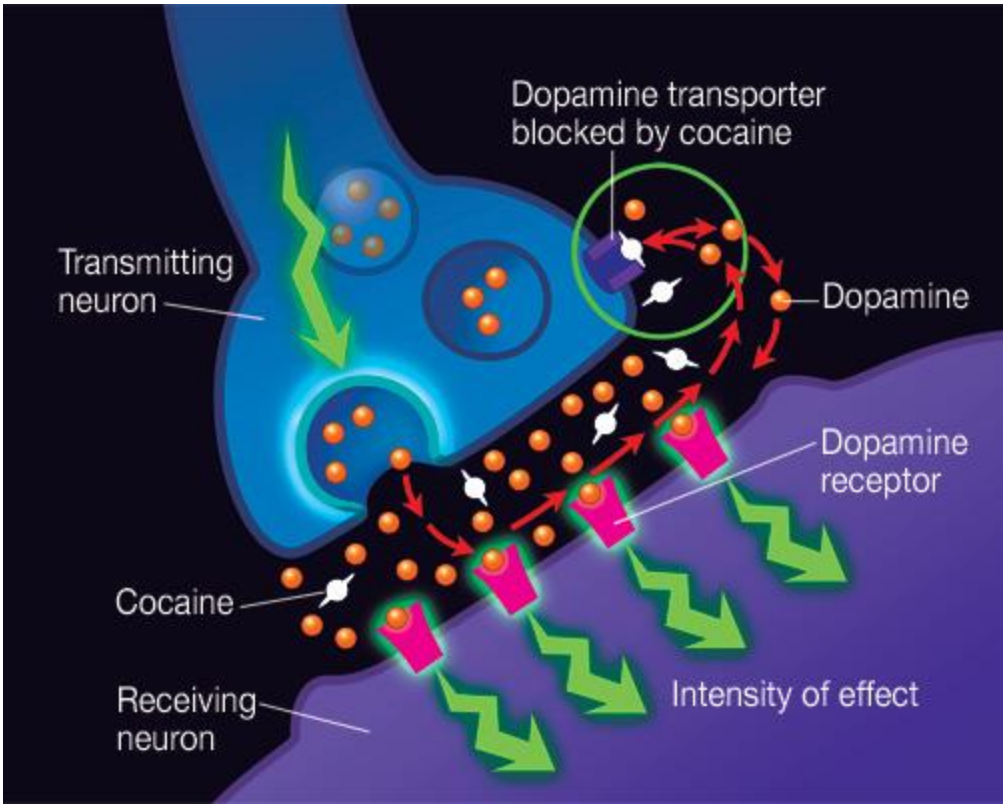

Figure 1.1 Depiction of the mechanism of action for cocaine
(modified from www.drugabuse.gov)

1.2. Opioid Receptor Pharmacology

Opioid receptors (the three classical opioid receptors are briefly detailed below) are a group of inhibitory G protein-coupled receptors (GPCR) that transverse through the cell membrane seven times. GPCRs interact and react to endogenous opioids that are distributed widely in the brain, and are found in the spinal cord and digestive tract. The endogenous opioids are dynorphins, enkephalins, endorphins, and endomorphins. When a ligand binds to the GPCR it causes a conformational change, which allows it to act as a guanine nucleotide exchange factor. The GPCR can then activate an associated G protein by exchanging its bound guanine diphosphate for a guanine triphosphate [7]. Effector systems activated or blocked upon opioid receptor G-protein interaction are adenyl cyclase, Ca^{2+} channels, K^{+} channels, or phosphoinositol turnover [8]. See figure 1.2.

GPCRs constitute a large protein family of receptors that sense molecules outside the cell and activate signal transduction pathways inside the cell [9]. The cyclic adenosine monophosphate (cAMP) signal pathway and the phosphatidylinositol signal pathway are the two principal signal transduction pathways of G protein mediated signaling [10]. For our purposes we will look exclusively at the cAMP pathway.

The cAMP-response element binding protein (CREB) proteins are activated by phosphorylation from various kinases, including protein kinase A (PKA). PKA regulates cell metabolism by phosphorylating specific enzymes in the metabolic pathway. PKA is activated when regulatory subunits are dissociated due to the conformational changes caused by the cAMP binding [11].

PKA then activates a CREB protein in the nucleus. The activated CREB protein then binds to a cAMP response element region. This binding activates the cAMP response element region,

allowing it to switch certain genes on or off. The endogenous ligand dynorphin is regulated by CREB proteins [11].

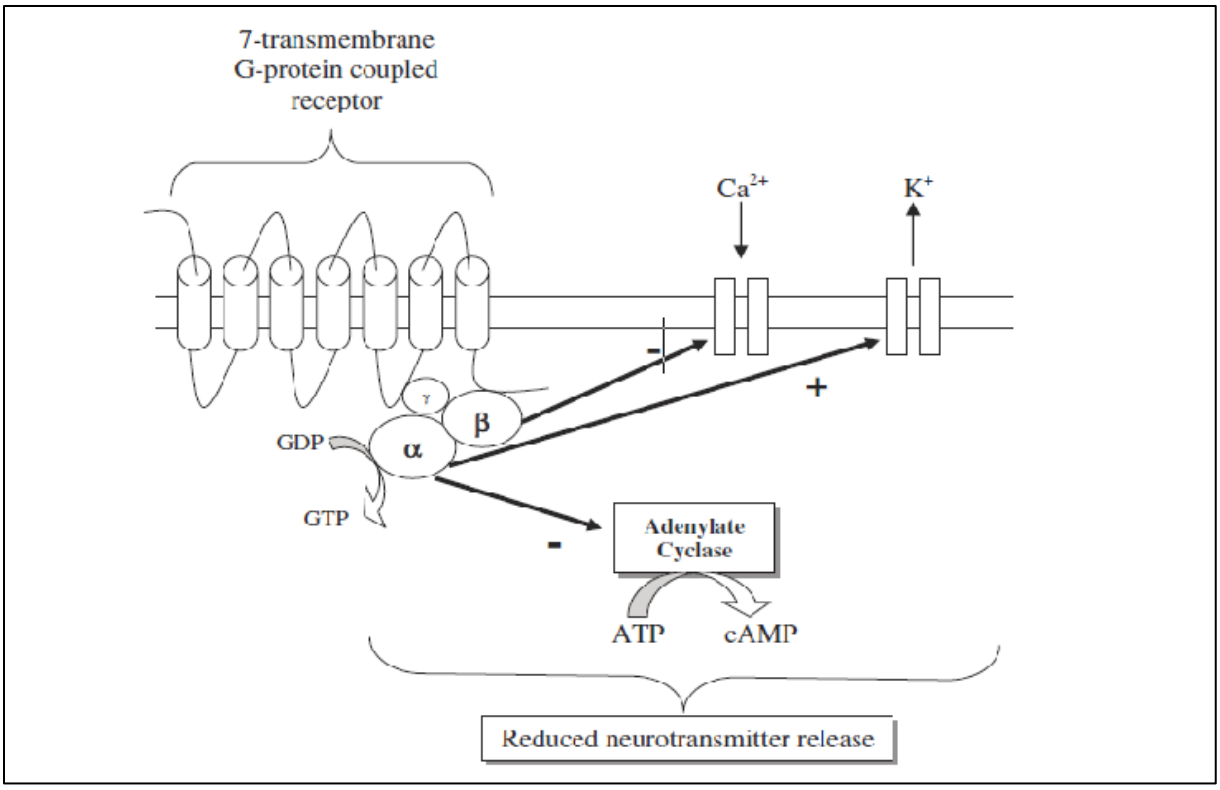

Figure 1.2 The seven transmembrane structure of opioid G-protein-coupled receptor. Receptor activation by opioid receptor ligands leads to initiation of intracellular transduction pathways that include stimulation of potassium efflux and inhibition of adenylyl cyclase. In this diagram the G-protein is denoted α, β, γ and the α-subunit interacts with K^+/Ca^{2+} channel and adenylate cyclase. Used with permission. [12]

1.2.1. *κ* opioid receptors (KOR)

The KOR is an opioid receptor that is located in the brain, spinal cord, and peripheral sensory neurons. In the brain it is found in the hypothalamus, periaqueductal gray, and claustrum. In the spinal cord it is found in the substantia gelatinosa [13]. The primary endogenous ligands that bind to the KOR are dynorphin A, dynorphin B, and α-neoendorphin [14]. The physiological functions of KOR is observed in analgesia, anticonvulsant effects, depression, dissociative/hallucinogenic effects, diuresis, dysphoria, miosis, neuroprotection, sedation, stress, and addiction control [13,14]. Due to the side effects of dysphoria, diuresis, and hallucinations KOR agonists have seen limited clinical utilization [15]. However, over the past decade KOR agonists have investigated for their therapeutic potential in the treatment of addiction [16]. The relationship between addiction and the KOR/dynorphin system will be a focal point of this work.

1.2.2. μ opioid receptors (MOR)

The MOR is an opioid receptor that is located in the brain, spinal cord, peripheral sensory neurons, and the intestinal tract. In the brain it is found in the laminae III and IV of the cortex, thalamus, striosomes, periaqueductal gray and rostral ventromedial medulla. In the spinal cord it is found in the substantia gelatinosa [13]. A high density of MOR receptors is found in the caudate putamen of the basal ganglia. MOR receptors are located presynaptically on primary afferent neurons within the dorsal horn of the spinal cord where they inhibit glutamate release and hence transmission of nociceptive stimuli [13]. The periaqueductal grey is an area of the midbrain involved in the central control of nociceptive transmission. The physiological functions of MOR can be observed in analgesia, physical dependence, respiratory depression, miosis, euphoria, reduced GI motility, and vasodilation [13,14]. The primary endogenous ligands that

bind to the MOR are the opioid peptides endomorphin, endorphin, met-enkephalin, and leu-enkephalin.

1.2.3. δ opioid receptors (DOR)

The DOR is an opioid receptor that is located in the brain and in peripheral sensory neurons. In the brain it is found in the pontine nuclei, amygdala, olfactory bulbs, and the deep cortex [13]. DOR utilize enkephalins as its endogenous ligands. DOR receptors are located presynaptically on primary afferents where they inhibit the release of neurotransmitters. The physiological function of DOR is seen in analgesia, antidepressants, convulsants, physical dependence, and respiratory depression [13,14]. The primary endogenous ligand that binds to the DOR are the opioid peptides met-enkephalin and leu-enkephalin.

1.3. Addiction and KOR

One of the leading views in understanding why the brain becomes addicted is that it strives to maintain equilibrium [17]. This concept is defined as allostasis, which is the process of achieving stability, or homeostasis, through physiological or behavioral change. Processes that are allostatic in nature are thought to alter homeostatic processes. An example of this would be frequent drug use changing the homeostatic equilibrium to include the drug. The result is a craving for the drug. Since the reward pathway is desensitized with repeated drug abuse, more drug is required to maintain homeostasis. Symptoms of drug withdrawal are then experienced when drug use has stopped and drug use has become drug abuse.

The KOR/dynorphin system is involved in the allostatic process. Drugs of abuse have been demonstrated to activate the CREB transcription factor. These effects on CREB have been associated with the modulatory increases in dynorphin and subsequent dysphoria-like behaviors due to KOR activation [18]. Overexpression of CREB in the nucleus accumbens has been shown

to decrease the rewarding effects of cocaine [19]. The dynorphin gene is known to be CREB regulated [11], and repeated cocaine administration increases its expression in the nucleus accumbens and dorsal striatum [20].

Dynorphin as a mediator of dysphoria-like behavior implicates activity of this system as one of the primary mediators of the anti-reward system following drug withdrawal, drug craving, and relapse to drug seeking [21]. KOR activation has been shown to inhibit evoked dopamine release in the nucleus accumbens [22], so that activation of this receptor reduces striatal dopamine transmission. See Figure 1.3.

Currently, the FDA has not approved a drug for the prevention of relapse due to cocaine addiction [23]. This has increased the interest of developing pharmaceutical agents that selectively bind to the κ opioid receptors (KOR) for the treatment of addiction [24]. Recent investigations have determined that KOR agonists exhibit a decreased liability for addiction as well as respiratory depression in comparison to MOR agonists [23]. Selectively binding antagonists to the KOR have only been utilized historically as pharmacological tools. Additionally, KOR antagonists, which penetrate the blood-brain barrier, show promising potential in therapeutic treatment of depression and anxiety, as well as opiate and cocaine addiction [23,25]. In rat models, co-administration of KOR antagonists with cocaine inhibited the induction of cocaine conditioned place preference [26]. This shows that a KOR antagonist can suppress dopamine release between synapses.

1.4. Zyklophin

KOR antagonists that are metabolically stable have been shown to penetrate the CNS. However, peptide-based KOR-selective antagonists are metabolized by proteases and are expected to have a shorter pharmacological activity than non-peptide KOR-selective antagonists

with increased metabolic stability. Through the incorporation of unnatural amino acids as well as conformational constraints in the C-terminus, zyklophin ([5,8] cyclic analogue [N-benzylTyr^{1},cyclo(D-Asp^{5},Dap^{8})]Dyn A- (1-11)NH_2) exhibits increased enzymatic stability against serum proteases [27]. The functionalities at positions 5 and 8 decrease affinity for the MOR and DOR resulting in elevated selectivity for the KOR. Low MOR affinity is due to the combination of N-benzylTyr^{1} with the [5,8] cyclicization. Without N-benzylTyr^{1} the relative affinity of KOR > MOR is only 9-fold. However, with the addition of N-benzylTyr^{1} the relative affinity of KOR > MOR is 194-fold [28].

Initial testing has shown that zyklophin crosses the blood brain barrier to antagonize the KOR in the CNS following *in vivo* administration. Zyklophin's duration of activity following a systemic dose is less than 12 hours, whereas a non-peptide KOR antagonists, such as nor-BNI, exhibits duration of activity up to 28 days after a single dose is administered. This prolonged activity of nor-BNI complicates its use as a pharmacological tool and as a potential therapeutic agent thereby increasing the importance of peptide-based KOR antagonists [23].

Peptide-based drugs have been shown to be successful pharmaceutical agents exhibiting high activity, high specificity, low toxicity, and minimal drug-drug interaction [29]. However, peptide-based drugs have been under-utilized due to low oral bioavailability. Due to modifications of the C-terminus and address domain, zyklophin has an increased metabolic stability, high KOR selectivity, and antagonist activity. Thus, zyklophin is a good lead compound for the development of potential treatments of cocaine and opioid addiction and relapse.

In this thesis work, zyklophin was subcutaneously administered to CD1 mice and the pharmacokinetics were investigated. Mice were sacrificed at specific timepoints post-dose and 1

mL of blood was collected via cardiac puncture. Following blood collection, the samples were immediately centrifuged and the plasma was collected. Then formic acid was added to the plasma sample to achieve a final formic acid concentration of 1%. Samples were then stored at -80°C until the time of analysis. The samples were analyzed via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to determine the concentration of zyklophin in each sample. In the following chapter, LC-MS/MS will be discussed as well as the results of the analysis of the plasma samples.

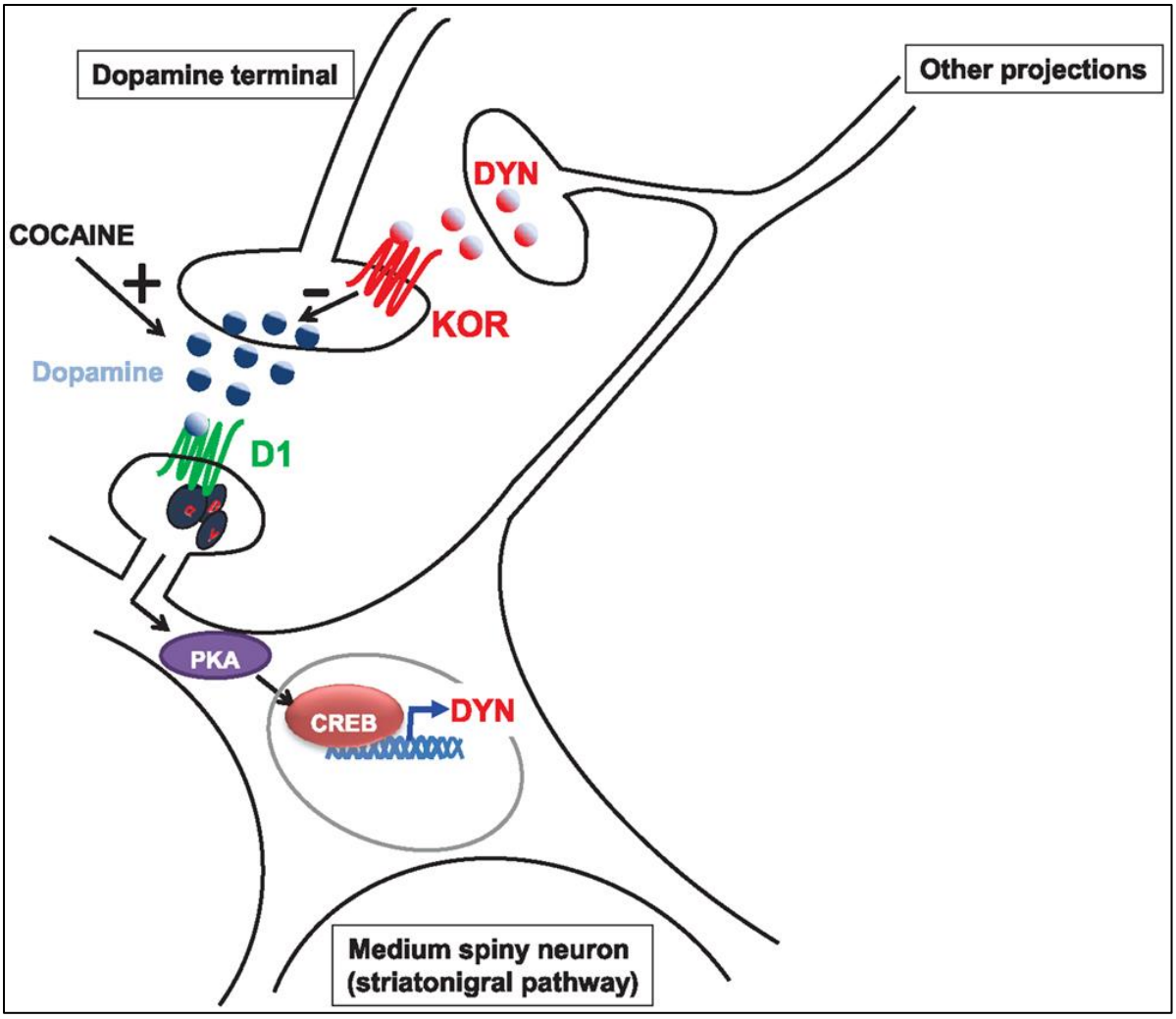

Figure 1.3 Model by which the dynorphin/kappa system could counteract cocaine-induced dopamine release. Cocaine administration elevates dopamine levels. Binding of dopamine on the D1 receptor activates the cAMP/PKA/CREB pathway which leads to dynorphin (DYN) synthesis. Binding of DYN to KOR located on dopamine terminals exert an inhibitory effect on dopamine release. Figure used with permission. [30]

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Chapter Two:

Development of an LC-MS/MS Method for the Quantitation of Zyklophin in Mouse Plasma

2.1 Introduction

Liquid chromatography in conjunction with triple-quadrupole tandem mass spectrometry (LC-MS/MS) is a highly effective method for the quantitation of peptides and proteins in a biological matrix [1]. It is widely utilized in industry as well as in academia. LC-MS/MS has been employed to investigate pharmacokinetics, pharmacodynamics and the stability of peptide-based pharmaceuticals [2,3]. There are many steps that are involved in the quantitation of a peptide in a plasma sample by LC-MS/MS. These include: sample preparation, liquid chromatography (LC) separation, analyte ionization, precursor ion selection, fragmentation, product ion selection and ion detection.

One of the more challenging problems that analytical chemists face is isolation of analyte(s) of interest from the sample matrix, in this case plasma [4]. Many problems can arise from a plasma sample that has not been properly prepared prior to injection into an LC-MS/MS platform. These problems include: matrix effects (i.e. ion suppression or enhancement), interferences, and development of high back pressures due to the instrument clogging and contamination. There are three main techniques that are employed for isolation of a peptide from its matrix for LC-MS/MS analysis: protein precipitation, liquid-liquid extraction, and solid phase extraction.

Liquid chromatography is a technique that can be used to separate each component in a sample mixture. When combined with a selective detector (e.g. tandem mass spectrometry) LC is a powerful tool that allows us to identify and quantify each component in the sample. A typical gradient LC system uses high pressure pumps to pass solvents of known composition (mobile phase) to an injector where a sample of unknown composition is injected onto a column that contains a packing material (stationary phase) that separates the analyte(s) based on their chemical and/or physical properties. For separation to occur each component of the sample,

including the analyte(s), must have a different affinity for the stationary phase. The separation is based on the equilibrium of the compound between the stationary phase and the mobile phase. If the interaction or affinity for the stationary phase is different for each component, then the elution of each component will occur at different times depending on how long each component spends in the stationary phase. All molecules spend the same amount of time in the mobile phase. There are many different types of stationary phases including polar, nonpolar, ionic and affinity supports. The analytical column used in this work separates compounds based on their hydrophobicity and will be discussed in greater detail below.

Upon elution from the column, the mobile phase carries the analyte to the ion source of the mass spectrometer. Ionization of the analyte occurs when the molecule acquires a positive or negative charge due to the gain or loss of a proton at the ion source. Upon reaching the source, the mobile phase is vaporized with a pressurized neutral gas (i.e. nitrogen or argon) at high temperatures. A voltage is then applied to cause ionization of the analytes of interest. This method is called electrospray ionization and will be discussed in greater detail below.

Once ionized, the analyte goes through the first quadrupole (Q1) of the triple-quadrupole mass spectrometer. In Q1, the analyte can exist in several charged states. One of the charge states is selected usually based on ion intensity and prevalence. This ion is then accelerated into the second quadrupole (Q2) using an electrical potential. In Q2, also called the collision chamber, the accelerated ion collides with neutral gas (e.g. nitrogen or argon) causing fragmentation. Upon fragmentation, the fragments are then accelerated into the third quadrupole (Q3). In Q3, there can be many different fragments produced from the original analyte. These are called product or daughter ions. As a result, product ion(s) are selected based on ion intensity and prevalence. Product ion selection and detection will be discussed below.

2.2 Sample Preparation Techniques in LC-MS/MS

Blood, plasma and serum contain proteases and other enzymes that can degrade proteins and peptides. In addition to this, peptides and proteins can undergo various pathways of chemical degradation such as oxidation, reduction, β-elimination and hydrolysis (typically of the peptide bond) [5]. To suppress enzymatic activity and chemical degradation, samples can be treated in various ways including: temperature control (e.g. sample handling on ice), pH control (acidifying samples with formic or hydrochloric acid), utilizing various coagulants (EDTA vs. sodium heparin), or the addition of specific enzyme inhibitors.

The presence of endogenous proteins in a sample can result in variable MS/MS and chromatographic behavior. More than 90% of the endogenous proteins found in plasma are comprised of only a few different high-abundance proteins. The major protein is serum albumin, which has a plasma concentration of 30–50 mg/ml. This accounts for approximately 60% of total serum protein [6,7]. The second most abundant proteins are immunoglobulins. When considering the presence of all immunoglobulins, they combine to account for up to 30% of plasma protein. Thus, achieving separation of the analyte from endogenous proteins prior to MS/MS is essential to avoid matrix effects or an overwhelming number of interfering peaks [6,7].

Endogenous proteins and peptides are difficult to eliminate from samples due to their chemical similarity to the target analytes. The 20 most abundant amino acids all possess the characteristic carboxylic acid and amine functional groups, and differ only by the group on the alpha carbon, which imparts the unique chemical characteristics. Most large peptides and proteins contain a mixture of all the amino acids, and therefore identifying analyte-specific chemical properties to exploit for sample preparation can often be challenging. As endogenous

proteins are usually much larger than therapeutic peptides and exhibit tertiary structure, the simplest way to eliminate proteins is through protein precipitation or size exclusion chromatography.

2.2.1 Protein Precipitation

A commonly utilized protein precipitation method is salt induced precipitation. At a low salt concentration, the solubility of proteins increases slightly. However, at high salt concentrations, the solubility of the proteins drops sharply causing the proteins to precipitate. High salt concentrations compress the solvation layer, which increases protein-protein interactions. As the salt concentration of a solution is increased, the charges on the surface of the protein interact with the salt, not the water. This causes the hydrophobic regions on the protein surface to become exposed causing the protein to precipitate out of solution. However, this method is not utilized with mass spectrometry due to the interference of the salt in analysis.

A second method of precipitation of proteins in plasma is by changing the pH of the sample solution. Precipitation occurs due to the effect of pH on the different functional groups on a protein. At the isoelectric point (pI), the net charge on the protein is zero. Proteins vary widely in their pI. To isolate a particular protein from solution, at pH = pI, the protein will no longer be soluble and will precipitate out of solution. The other option is to use extremes in pH. At low or high pH values, the tertiary structure of the protein is altered due to the changes in electrostatic interactions, causing the protein to precipitate. This can be accomplished by the addition of an acid or base to a sample to denature the proteins.

A third method is the addition of a water-miscible organic solvent. Organic solvents precipitate proteins by reducing the dielectric constant of the medium. Organic solvent molecules usually bind to specific hydrophobic residues on the protein molecule. This disrupts the

hydrophobic interactions that maintain the protein's tertiary structure leading to precipitation. In this approach, precipitation involves mixing the plasma or serum sample with a water-miscible organic solvent (often acetonitrile or methanol) to achieve an organic solvent composition of approximately 75% [8]. Endogenous proteins are precipitated and can be removed from the sample by centrifugation. Proteins and peptides precipitate at varying concentrations of organic solvent. Large proteins (>100 kDa) typically precipitate at >30% solvent, whereas smaller proteins usually require a greater proportion of solvent to precipitate, and small peptides often do not precipitate at all [1].

When using acetonitrile, as little as a 50% final solvent composition still achieves >90% precipitation. Acetonitrile has been shown to be a superior organic plasma protein precipitant at volume ratios <2:1 (precipitant:plasma) [9]. This half-aqueous precipitation has been reported to help maintain the solubility of small polar peptides, while precipitating larger endogenous interferences [8]. In contrast, peptides generally do not precipitate at all with organic solvents as they are small and lack significant tertiary structure. Peptides smaller than approximately 2.5 kDa tend to behave similar to small polar molecules with respect to their extractability and LC–MS/MS behavior, although they often demonstrate a stronger tendency for adsorption, insolubility and protein binding [1].

Upon protein precipitation, the sample is centrifuged and the supernatant is extracted. Depending on the mobile phase utilized in the LC method, the extracted aqueous layer may be ready for injection into the LC. However, if the final sample solution is incompatible with the desired mobile phase or analytical column, further sample preparation steps will be required. In this case, to achieve a desirable final sample solution composition, the sample can be dried under inert gas (e.g. nitrogen or argon) or under vacuum and then reconstituted in a solution that is

more compatible with the selected mobile phase and analytical column. Depending on the difficulty of ionization and the sensitivity of the method, these dried samples can be reconstituted in smaller volumes to achieve higher concentrated samples to analyze.

2.2.2 Liquid-Liquid Extraction

Liquid-liquid extraction (LLE) is also known as partitioning or solvent extraction. LLE is a technique in which compounds are separated based on their relative solubility in two immiscible liquids. For our purposes we will consider water and a water-immiscible organic solvent. Since water is very polar, a non-polar organic solvent, such as methyl tert-butyl ether or n-butyl chloride, should be chosen. The distribution or partition coefficient (K) is a quantitative measure of how a compound will distribute between an aqueous and organic phase. This coefficient is the ratio of the solubility of the dissolved solute. Essentially, K is the ratio of concentrations of the solute in the two different solvents once the system reaches equilibrium. The larger the value of K, the more selective a solution will be for any given compound.

Following the addition of the organic solvent and mixing of the two layers, the sample is centrifuged to separate the two liquids. Removal of the organic layer can be accomplished in two ways. One method is to manually remove the organic layer by pipetting. A second method is to rapidly freeze the aqueous layer, typically in a dry-ice acetone bath. Following separation of the organic solvent layer, the solvent is evaporated under inert gas or vacuum and the residue is reconstituted with a solution that is compatible with the mobile phase and analytical column used for the liquid chromatography separation. However, it should be noted that LLE is a poor method for the isolation of peptides and proteins as the organic solvents employed can cause

denaturation of the larger peptides and proteins. Only highly hydrophobic membrane bound peptides would be extracted in LLE.

2.2.3 Solid Phase Extraction

Solid phase extraction (SPE) is a sample preparation method that separates components that are present in a solution according to their physical and chemical properties. SPE utilizes a sorbent that exhibits specific properties that result in selective binding with the solute of interest. As a result, as solution is passed through the sorbent, the solute of interest will remain within the sorbent structure while impurities of the solution will pass through. There are many types of SPE sorbents. These include ion exchange, size exclusion, normal phase, hydrophilic interaction, reversed-phase, and HLB (a polymeric sorbent containing both hydrophobic and hydrophilic character).

In this work the polymer-based reversed-phase Oasis HLB 96-well Plate, 30 mg sorbent per well, 30 µm particle size sorbent was selected due to its optimal sensitivity for therapeutic peptide analysis and reduced matrix effects which yields greater selectivity. The steps involved in reversed-phase SPE include but are not limited to: conditioning/wetting sorbent with, a wash step, a loading step, a wash step, and elution. In the conditioning step a polar solvent is added to the column in order to wet the sorbent, allowing for efficient wetting of the surface. Following the conditioning step, the sorbent is washed with water to remove all traces of the solvent and to wet the silica surfaces that were penetrated by the solvent. At this point, the sample is then loaded and passed through the column. Upon loading, the column will be washed several times with various solutions, usually an aqueous or a low organic solvent composition solution, to clean the column of hydrophilic impurities in the sample.

Following the washing steps, the solute of interest can be eluted off the column with a solution containing a high percentage of organic solvent. If the final eluted sample solution is incompatible with the mobile phase or analytical column used for the separation, further sample preparation steps will be required. To achieve a final sample solution composition, the solvent can be evaporated under an inert gas or a vacuum and then reconstituted in a smaller volume of a different solution that is more compatible with the selected mobile phase, analytical column, and to improve the limit of detection. The specific method developed for these studies is found in Appendix 1.

2.3 Liquid Chromatography (Reversed-phase)

Due to the compatibility with plasma samples and aqueous solutions, reversed-phase liquid chromatography is ideal for the separation of biological samples. Reversed-phase liquid chromatography exhibits less peak tailing than normal phase chromatography because the stationary phase has fewer polar or anionic sites that can strongly adsorb a solute. As a result, separation is also less sensitive to polar impurities in the mobile phase than the stationary phase utilized for normal phase chromatography. A C-18 column was chosen for these studies based on its use in previous studies regarding dynorphin peptides [10].

The separation mechanism in reversed phase chromatography depends on the hydrophobic interaction between the solute molecule present in the mobile phase and the stationary phase of the analytical column. The mobile phase conditions used in reversed phase chromatography are primarily aqueous. Retention on the column is dependent on interactions between the analyte and the hydrophobic stationary phase. Very hydrophilic components of the sample do not interact with the stationary phase and elute in the void volume.

Reversed phase chromatography is an adsorptive process that relies on a partitioning mechanism to effect separation. The analyte molecules partition and establish equilibrium between the mobile phase and the stationary phase. The distribution of the analyte between the two phases depends on the binding properties of the stationary phase, the hydrophobicity of the solute and the composition of the mobile phase. Initial mobile phase compositions are selected to favor adsorption of the analyte of interest from the mobile phase to the stationary phase. For both isocratic and gradient elution, upon absorption of the analyte, the mobile phase composition is modified to favor desorption and elution of the analyte from the stationary phase back into the mobile phase. In this case, adsorption is considered the extreme equilibrium state where the distribution of solute molecules is essentially 100% in the stationary phase. Conversely, desorption is an extreme equilibrium state where the solute is essentially 100% distributed in the mobile phase [11].

Reversed phase chromatography of biomolecules, especially in biological samples, generally requires gradient elution instead of isocratic conditions and, this, therefore, will be the focus of this work. In general, peptides and proteins adsorb to the surface of a reversed-phase stationary phase under aqueous conditions. However, they desorb from the stationary phase within a very narrow window of organic modifier concentration. As a result, the concentration of organic solvent passing through the column needs to be controlled to avoid the simultaneous elution of the analyte with the impurities and potential interferences. Thus, gradient elution is the only practical method for reversed phase separation of complex biological samples [12].

The structure of a peptide should be carefully considered in order to select appropriate initial mobile phase gradient conditions. After determining which mobile phases are most suitable for peptide analysis, the first step in the chromatographic process is to equilibrate the

column under suitable initial mobile phase conditions of pH, polarity, and ionic strength. The peptide is then eluted by decreasing the polarity of the mobile phase through the addition of organic solvents, such as acetonitrile. The initial mobile phase composition must be nonpolar enough to dissolve the partially hydrophobic analyte but polar enough to ensure binding of the solute to the reversed phase chromatographic matrix of the analytical column. Once the column is equilibrated with the correct composition of mobile phases A and B, the sample is injected into the analytical column where it interacts with the stationary phase. See Figure 2.1 for a depiction of the instrument set-up for gradient elution.

The next step of the reversed-phase chromatographic process is desorption and elution of bound solutes from the analytical column by decreasing the polarity of the mobile phase. Decreasing the polarity is accomplished by gradually increasing the percentage of organic solvent in the mobile phase by mixing mobile phase A and B. At a specific composition of mobile phases A and B, the sample will elute off the column. The gradual decrease in mobile phase polarity is achieved by increasing the organic composition of the overall mobile phase by increasing the percentage of B. This gradual change in mobile phase composition causes a decrease in polarity. As a result, the bound solutes in the column desorb and elute in order of their respective hydrophobicity.

The next step is the washing of the column with a high percentage of mobile phase B over a period of time. This step ensures that all lipids and hydrophobic peptides and proteins and potential interferences are washed off the column before the injection of the next sample. The final step of the chromatographic process is re-equilibration of the LC column. This final step is important because it removes residual organic solvent that could otherwise cause poor retention of the following sample injection as interference or elevated background noise. Since this can be

a time consuming process, mobile phases A and B are typically chosen so that they only differ in regards to organic solvent composition to allow for a more rapid and efficient re-equilibration. See Figure 2.9 for gradient profile descriptions utilized in this work.

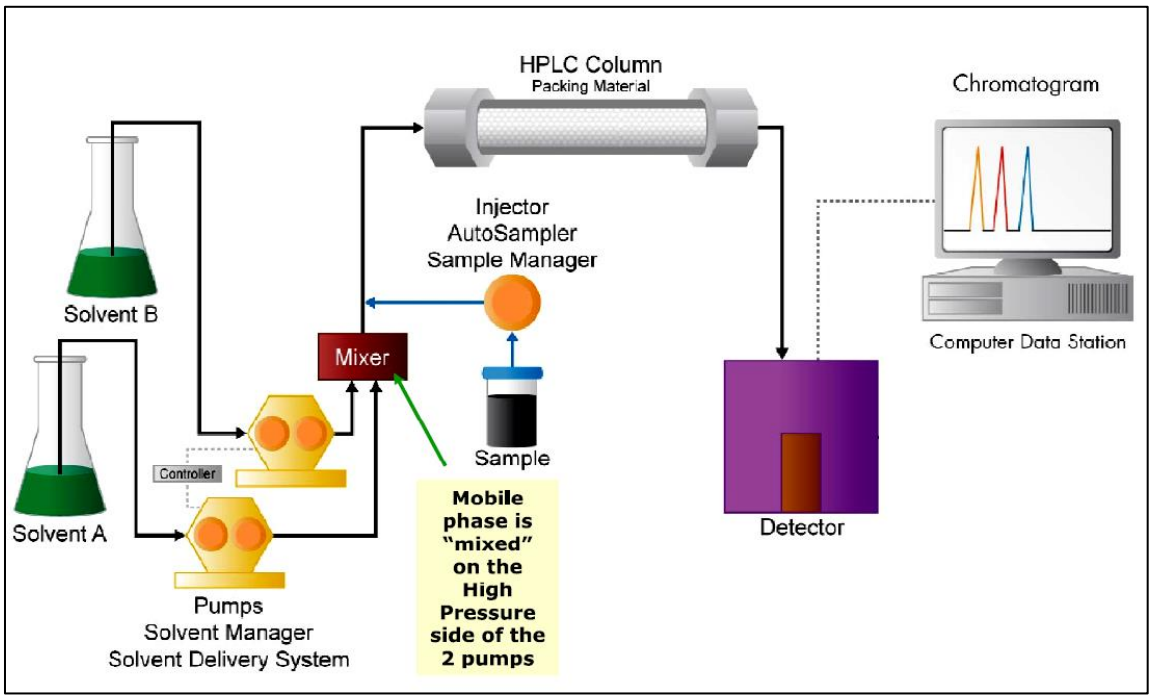

Figure 2.1: Gradient elution instrumentation depiction. Modified from Waters website (www.waters.com).

2.4 Electrospray Ionization

Electrospray ionization (ESI) makes it possible to use liquid chromatography in conjunction with mass spectrometry. ESI is considered a soft ionization technique because it does not typically cause macromolecules to fragment but causes formation of charged droplets containing analyte. It can be utilized in either positive or negative ion mode. Following the formation of charged droplets in positive ion mode, the droplets are further desolvated into smaller droplets containing newly attached protons. Charged compounds then move to the surface of the droplet to minimize Coulombic repulsion. As this occurs, the ions are vaporized and then pass through the mass analyzer and into the detector. See Figure 2.2 for a diagram of the ionization process.

2.5 Precursor/Product Ion Selection and Fragmentation

A triple quadrupole mass spectrometer consists of two quadrupoles in series with a non-mass resolving quadrupole in between to serve as a cell for collision-induced dissociation. The MS experiments of interest in this work are precursor ion scans, product ion scans and multiple reaction monitoring. Precursor ions are selected in the first quadrupole (Q1). The ions are then accelerated into the collision cell (the second quadrupole), where they are fragmented by colliding with nitrogen or argon gas molecules. Upon fragmentation the ions then enter the third quadrupole (Q3) where the product ion is selected and detected.

The product ion of interest is isolated in Q3 according to its mass-to-charge ratio (*m/z*). The quadrupole mass analyzer can also be considered as a mass filter. Direct current (DC) and radio frequencies (RF) applied in the quadrupole affect the trajectory of all ions in Q3. Both the DC and RF voltages are optimized to allow only ions with a specific m/z to pass through. All other ions will not have a stable trajectory through the quadrupole mass analyzer and will collide with the quadrupole rods and never reach the detector.

In these studies, precursor and product ion scans were performed to optimize the instrument parameters for our analyte of interest. From the information obtained from these scans, multiple reaction monitoring (MRM) was utilized to compare multiple daughter ions for their sensitivity, quantitation, and selectivity. See Figure 2.3 for a diagram of precursor and product ion scanning and multiple reaction monitoring.

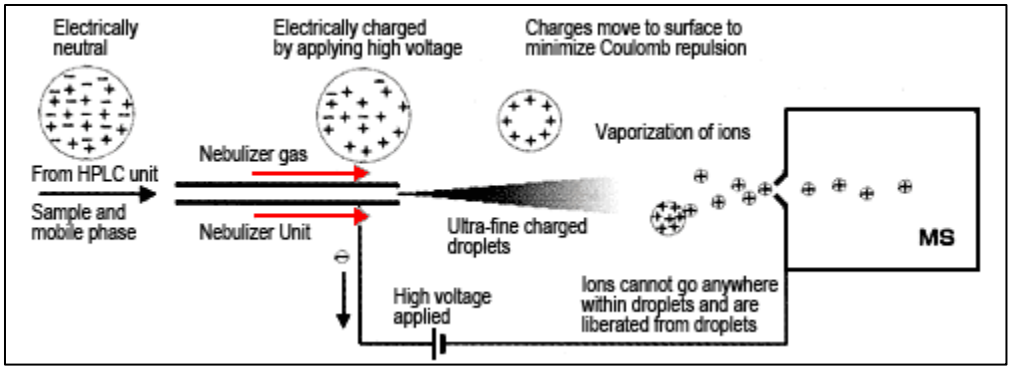

Figure 2.2 Depiction of ESI. Modified from Shimadzu website (www.shimadzu.com).

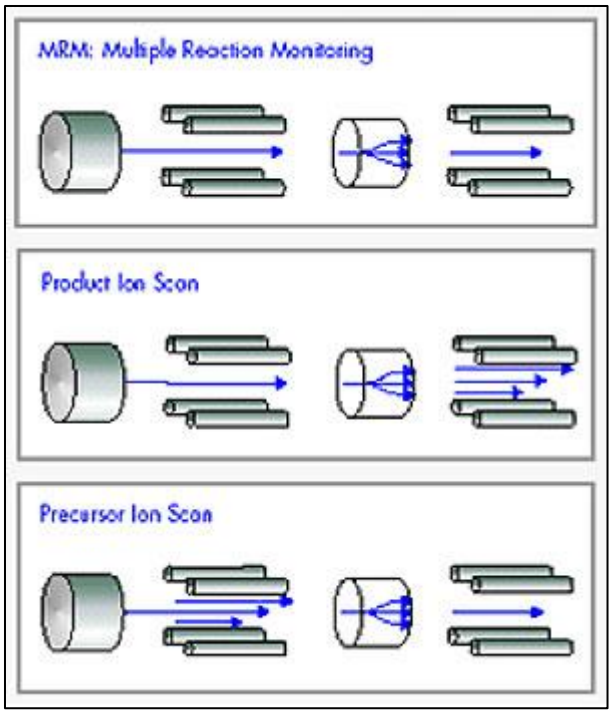

Figure 2.3 Depictions of precursor ion scan, product ion scan, and multiple reaction monitoring.

Modified from Waters website (www.waters.com).

2.6 Materials and Experimental Methods

2.6.1 Materials

Zyklophin and the structurally related N-methyl tyrosine dynorphin (1-11) analog (Figure 2.4), which was used as the internal standard, were synthesized in Professor Jane Aldrich's laboratory as reported previously.[11] HPLC-grade acetonitrile and Dulbecco's phosphate buffered saline (PBS) were obtained from Fischer Scientific (Pittsburgh, PA, USA), and deionized water was obtained from a Milli-Q Synthesis A10 Water Purification System (Millipore, Billerica, MS, USA). Formic acid was purchased from Acros Organics (Morris Plains, NJ, USA) at 99.9% purity.

2.6.2 Animals

Adult male CD1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in accordance to the National Institute of Health Guide for Care and Use of Laboratory Animals. Adult male CD1 mice, weighing 30-35g, were administered zyklophin by the subcutaneous route at 3 mg kg^{-1} in PBS. The dosing solution (0.100 mL) for the subcutaneous administration was geared to the weight of the mice. Animals were sacrificed at 15, 30, 60, 120, 240, 480 and 720 minutes post-dose, and 1.0 mL of blood was obtained via cardiac puncture. The blood samples were immediately centrifuged at 15,000 rpm for 15 min to harvest plasma. The samples were kept on dry ice and processed on ice. Prior to freezer storage samples were treated with formic acid to achieve a final concentration of formic acid of one percent.

The long-term stability of zyklophin in plasma was conducted at Celerion (Lincoln, NE). Plasma samples were prepared in CD1 mouse plasma supplied by BioreclamationIVT and were fortified with formic acid to achieve a final formic acid concentration of one percent. Six

replicates at 10 and 1000 ng/mL were tested following a 2 month storage at -80°C. Relatively small losses of zyklophin were observed at 10 ng/mL (11 ± 1%) or 1000 ng/mL (2.5 ± 0.7%).

2.6.3 LC-MS/MS Analysis

Liquid chromatography was performed using an ACE C18 column (5μm, 50 x 3.0 mm), supplied by MAC-Mod, with a flow rate of 0.2 mL/min and an injection volume of 30 μL using a Waters Acquity Classic UPLC (Waters Corp., Milford MA) coupled to a triple quadrupole mass spectrometer (Quattro Ultima Micromass Ltd. Manchester UK) operating in the positive-ion mode. The peptides were separated using the following gradient of aqueous 0.08% formic acid in water (mobile phase A) and acetonitrile containing 0.08% formic acid (mobile phase B): 0 min (1% B), 5 min (60% B), and 7 min (1% B). From time 0 min to 5 min the %B was increased with a profile curve of 9. From time 5 min to 7 min the %B was decreased with a profile curve of 6 (see Figure 2.9).

The precursor ions of zyklophin $[M+3H]^{3+}$, *m/z* 470.5, and its internal standard $[M+4H]^{4+}$, *m/z* 345.0, were first isolated and then subjected to collision-induced dissociation to give their fragment ions. Data acquisition was carried out with Mass Lynx 4.1 software with the following settings: capillary voltage, 2800 V; cone voltage, 25 V; source temperature, 100 °C; desolvation temperature, 200°C; cone gas flow, 82L/h; desolvation gas flow, 1157 L/h. Q1 and Q3 resolution were 0.8uFWHH. The argon filled collision cell pressure was 1.63×10^{-3} mbar on a gauge in-line with the cell. Multiple-reaction-monitoring was used for zyklophin $[M+3H]^{3+}$ *m/z* 470.5 $\rightarrow$ 91.2 and 226.3, and for the internal standard $[M+4H]^{4+}$ *m/z* 345.0 $\rightarrow$ 150.2 with a collision energy of 25 eV and dwell time of 0.3 s (see Figure 2.3).

2.6.4 Sample Preparation

Stock and working solutions of zyklophin and internal standard were prepared in polypropylene tubes with Milli-Q water. The calibration standards were prepared freshly in blank (drug-free) mouse plasma using the zyklophin working solution. The plasma calibration curve consisted of: zero standard, 10, 15, 25, 50, 100, 200, 500, and 1000 ng/mL. See Figure 2.5. To a mixture of 25µL of ice-cold plasma and 25µL of ice-cold internal standard (500 ng/mL prepared in ultrapure water) was added ice-cold acetonitrile (200µL) to precipitate the proteins out of solution. Following the precipitation, samples were vortexed and centrifuged at 10,000 rpm for 10 minutes. Immediately following centrifugation, 150µL of the supernatant was dried down in a speed vacuum concentrator. The residue was then reconstituted in 75µL of 0.1% formic acid and analyzed by LC-MS/MS according to the method described above.

2.6.5 Results and Discussion

Previous attempts by Professor Aldrich's lab to determine the pharmacokinetic properties of zyklophin by LCMS/MS were unsuccessful. The main obstacles encountered were irreproducibility of retention times, lack of resolution of zyklophin, and undesirable peak shape. This was believed to be due to the column employed during LC-MS/MS analysis and possible interaction with other components in the sample (e.g. lipids) that affected the column. In these studies, protein precipitation by acetonitrile proved to be a quick efficient method for sample cleanup prior to LCMS/MS analysis. Acetonitrile was selected due to its high efficiency at precipitating proteins in mouse plasma [9]. As seen in Figure 2.5, the standard curved showed good linearity resulting in good quantitation of the plasma samples.

Retention and peak shape issues were resolved by using a different reversed phase column and changing the elution following injection into the LC system. In this work, an isocratic approach was initially tested, but did not provide adequate separation or retention in

various compositions of acetonitrile and ultrapure water. Next, a convex gradient approach was attempted where the composition of B would rapidly replace A. However, as with an isocratic LC method, adequate separation and retention was not achieved. Finally, a concave gradient approach (see Figure 2.9) was employed where the composition of B slowly replaces A.

As seen in Figure 2.4, two product ions channels for zyklophin were evaluated during quantitation. The product ion with an *m/z* of 91 was utilized for quantitation due to the consistent reproducibility of this ion's prescence throughout multiple samples. The product ion with an *m/z* of 226 did not exhibit consistent intensity throughout the multiple sample run. Additionally, as seen in Figure 2.6, a peak at the retention time of zyklophin is present in the zero standard. This peak, along with the lack of reproducibility of the 226 ion, indicate that there is a matrix effect that is affecting its analysis and quantitation.

See Figure 2.8 for comparison of chromatograms of zyklophin 15 minutes post 3 mg/kg subcutaneous injection. The top chromatogram is from this work and the bottom chromatogram is from the previous attempt to characterize the pharmacokinetic properties of zyklophin. As can be seen from Figure 2.8, peak resolution is greatly increased, resulting in reliable quantitation with an LLOQ of 10 ng/mL. The method described in this chapter was then applied to investigate the pharmacokinetics of zyklophin following subcutaneous administration. The results of theses studies are given in Chapter 3.

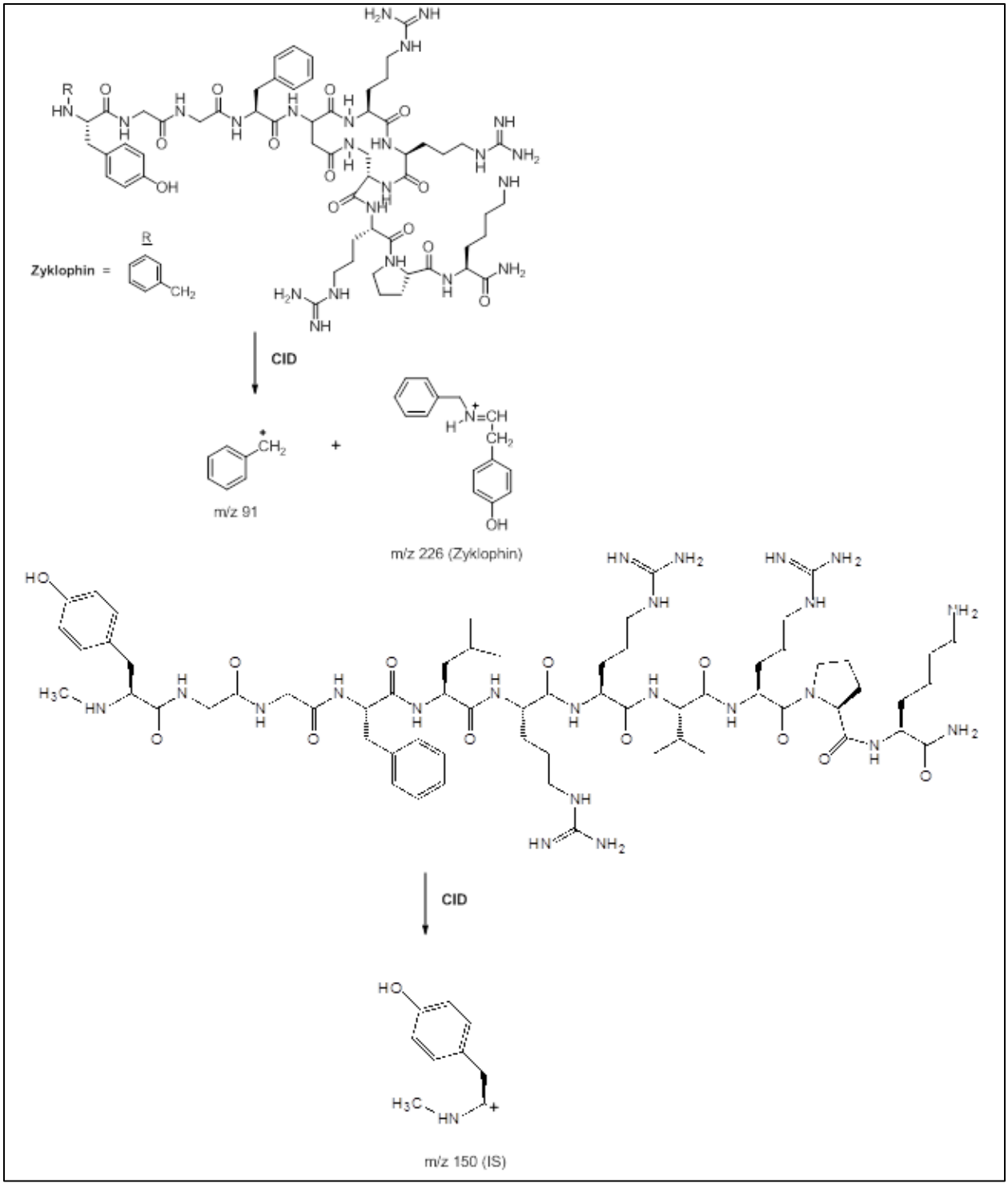

Figure 2.4 Structure of zyklophin (top structure) and internal standard (bottom) ([NMe-Tyr^{1}]dynorphin A-(1-11) amide) precursor and product ions

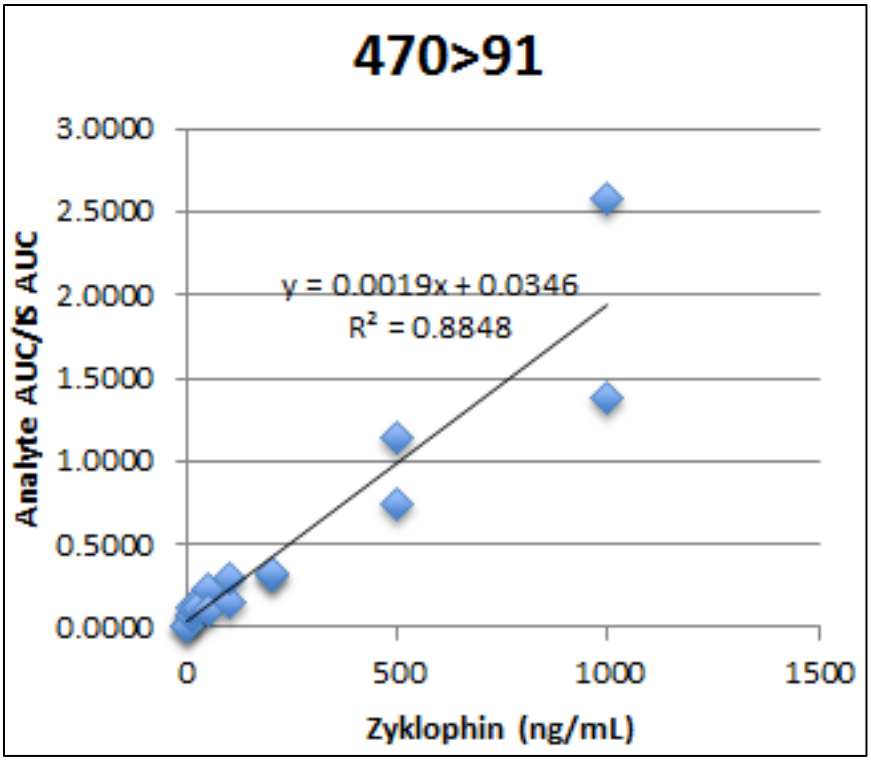

Figure 2.5: Standard calibration curve of zyklophin in mouse plasma

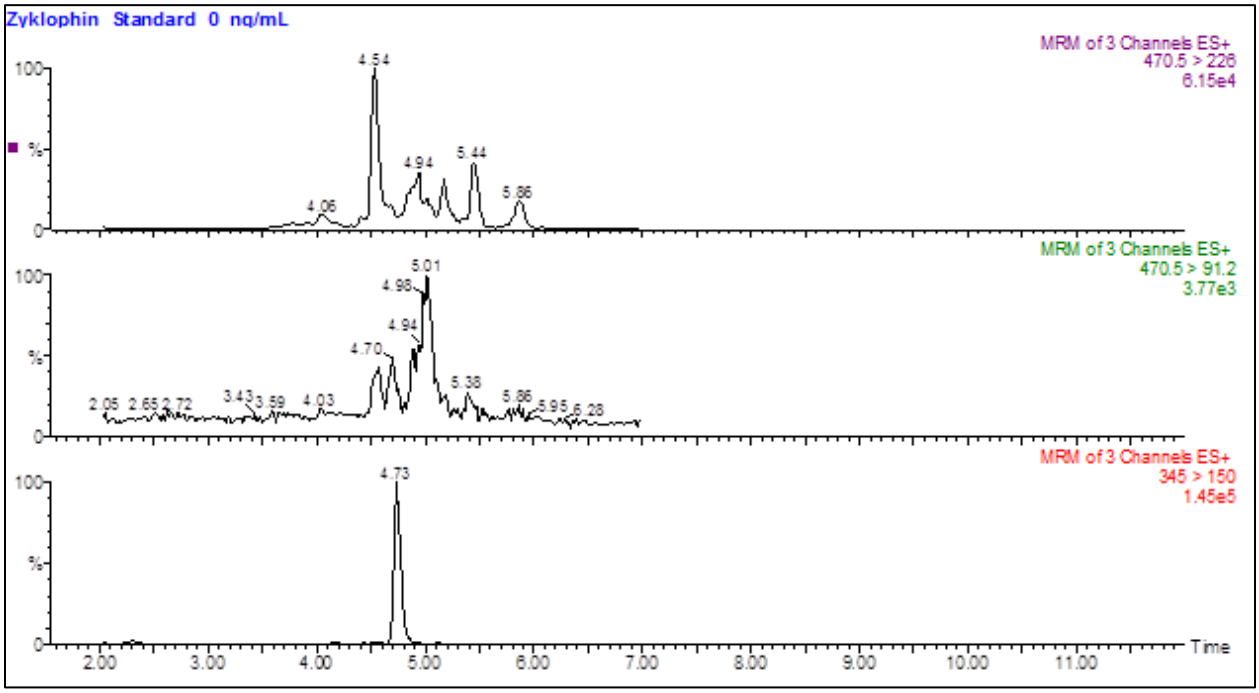

Figure 2.6: Blank sample chromatogram for both zyklophin product ions and the internal standard

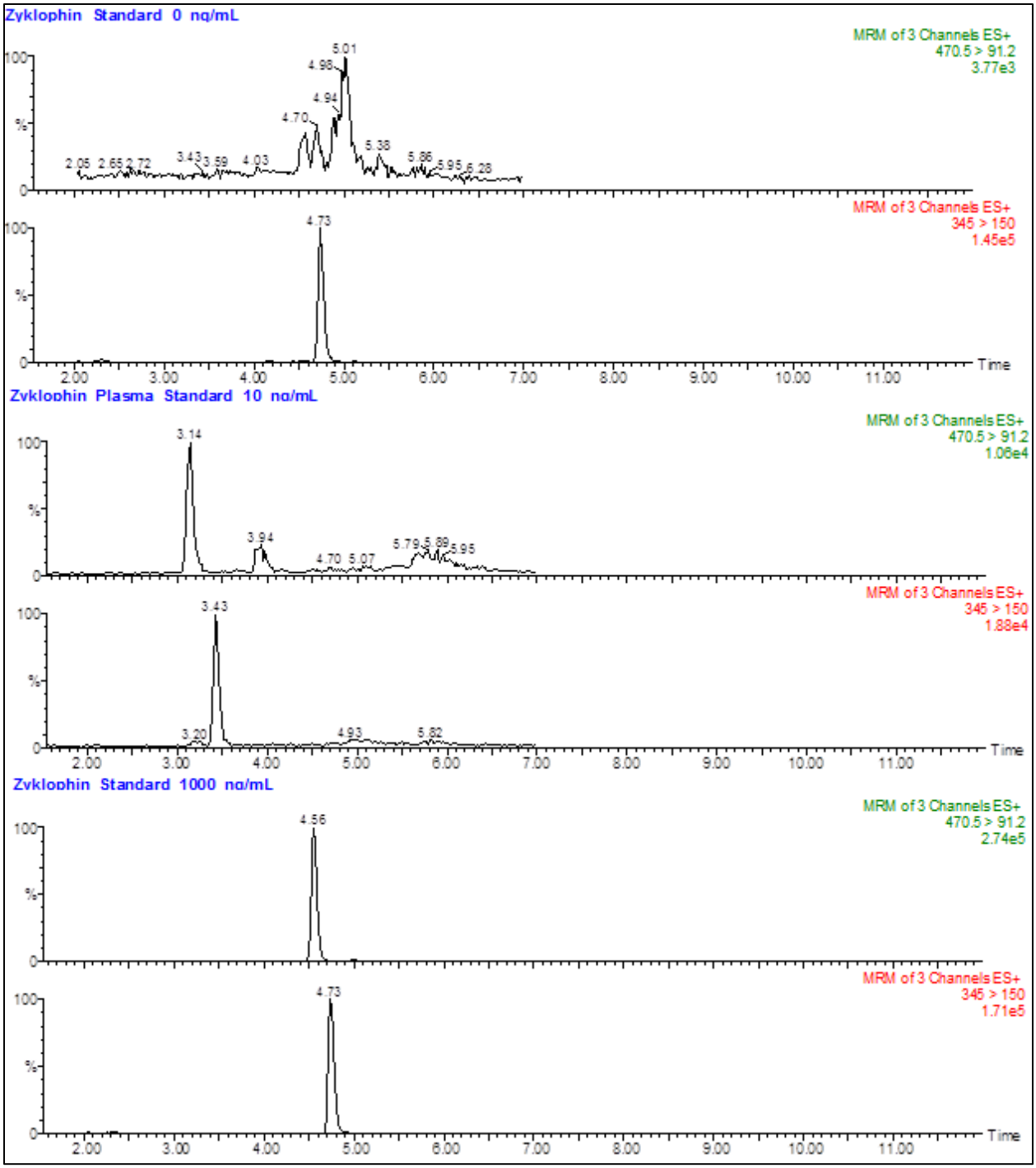

Figure 2.7: Selected zyklophin standard chromatograms at 0, 10, and 1000 ng/mL. (The retention time of zyklophin is typically 4.5-4.6 minutes and the IS retention time is typically 4.7-4.8 minutes; the reason for the earlier retention times for the 10 ng/mL standard is not known.)

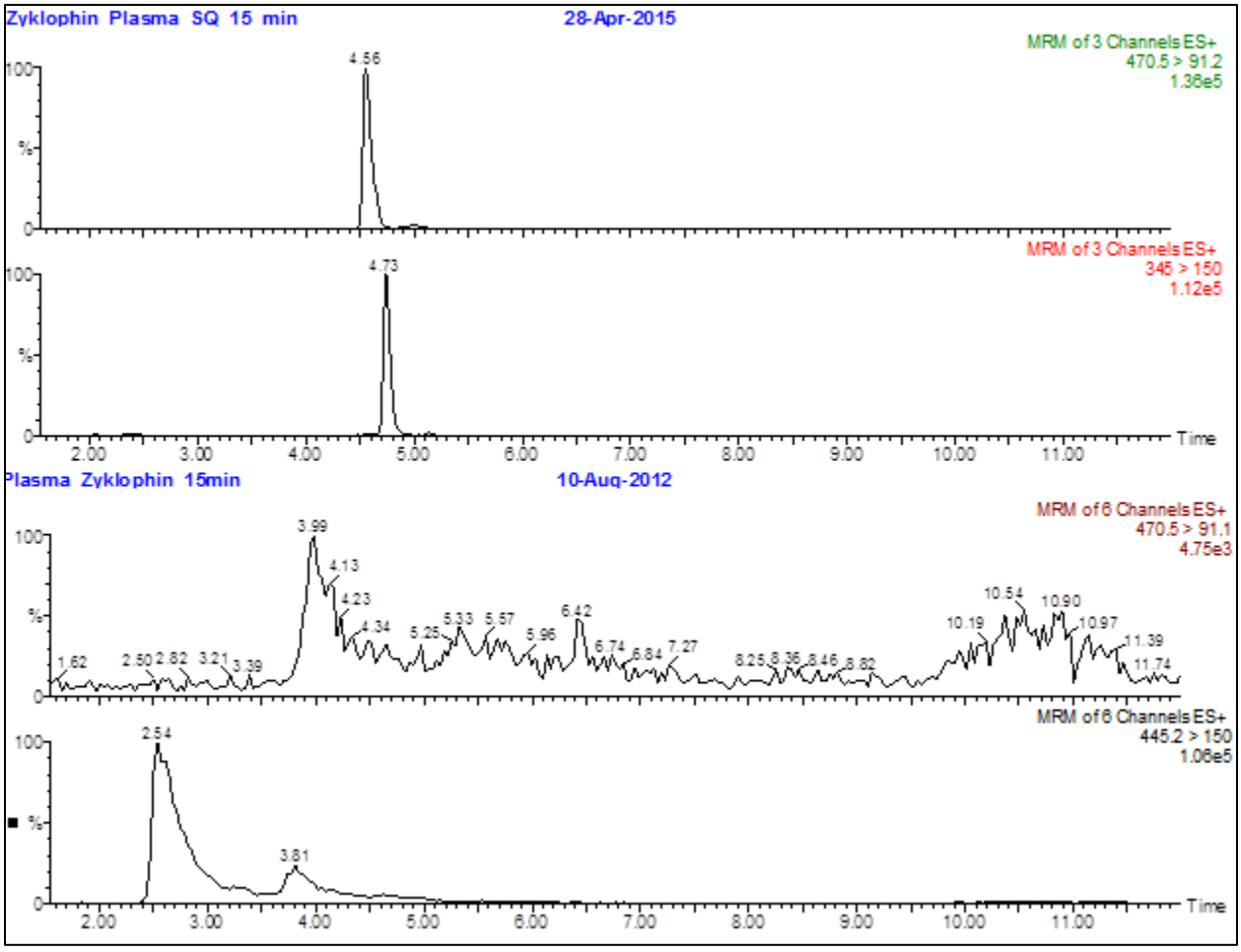

Figure 2.8: Comparison of chromatograms following 3 mg/kg subcutaneous injection. The top chromatogram is from this work. The bottom chromatogram (provided by Dr. Jane Aldrich's lab – University of Florida) is from the previous attempt to characterize the pharmacokinetic properties of zyklophin.

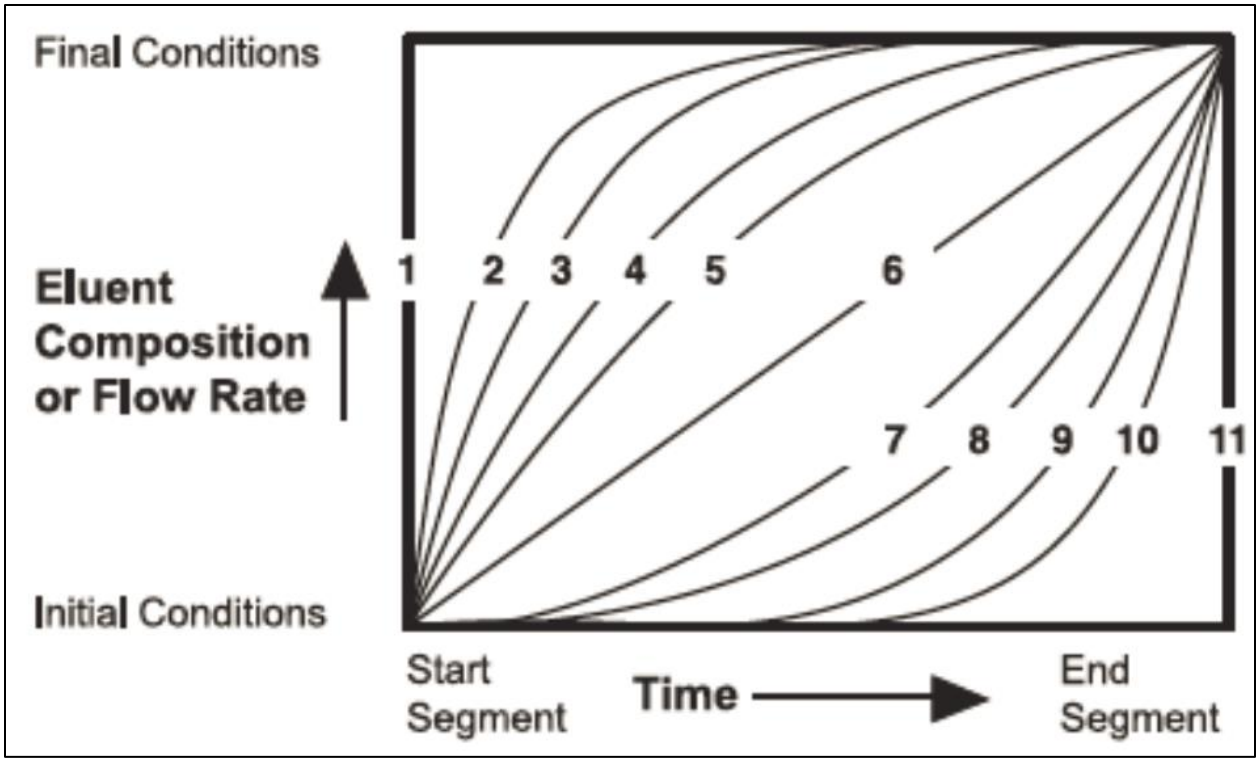

Figure 2.9: Gradient elution profiles. In this work, a concave (9) profile was used for the increase of % B in the mobile phase from time 0 minutes (1% B) to time 5 minutes (60% B). A linear (6) profile was used for the decrease of % B in the mobile phase from time 5 minutes (60% B) to time 7 minutes (1% B).

2.7 References

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Chapter Three:

Pharmacokinetics of Zyklophin via Subcutaneous Administration

3.1 Introduction to Pharmacokinetics

The field of pharmaceutics is concerned with factors that influence the stability of a drug within the drug product, the release of drug from the drug product, the rate of dissolution and release of the drug at the adsorption site, and the delivery of drug to the site of action. Drug products are dosage forms with specific drug delivery systems that release and deliver the drug to the site of administration. Pharmaceuticals are substances intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease. Pharmaceuticals can be administered via a variety of dosage forms. The different types of dosage forms include capsules, tablets, liquids, and suspensions. Drug products are evaluated for safety, efficacy, and convenience for the targeted population. Biopharmaceutics is a field that investigates the relationship between the physical/chemical properties of a drug, drug product and route of administration on the rate and extent of drug absorption.

As mentioned above, drugs can be delivered via various routes of administration. The drug is released from the drug product following administration and a fraction of the drug is then absorbed into surrounding tissue. This can either be directly from the site of administration (subcutaneous) or in the GI tract (oral). It then enters the bloodstream and once it reaches the site of action, a pharmacologic response results as long as the drug concentration is greater than or equal to the minimum effective concentration.

In order to investigate a new drug substance, drug bioavailability is evaluated following administration by different routes in animal subjects prior to human studies. Pharmacologic, toxic, and clinical responses for the different doses and modes of administration are assessed in the initial experiments. Drug bioavailability can differ widely depending on the formulation,

even for the same route of administration. As a result, therapeutic effectiveness can vary between different formulations containing the same drug. The chemical properties of the drug molecule, route of administration, identity of metabolites and the degree of metabolism can determine whether an administered drug is therapeutically effective, toxic, or has no observable effect.

The US Food and Drug Administration (FDA) must approve all drug products that intend to be marketed in the United States. Therefore, pharmaceutical manufacturers are required to perform extensive development and clinical research of drug effects in both animals and humans prior to approval. Biopharmaceutics provides the scientific basis for peptide, protein, and oligonucleotide based drug product design and development. Studies of biopharmaceuticals use both *in vitro* and *in vivo* methodologies. *In vitro* methods do not involve the dosing of animals or humans. *In vivo* methods are complex studies that test drug exposure within living animals and humans. In this work, a mouse model was used to determine the bioavailability of zyklophin following subcutaneous administration.

As mentioned before, after drug is released from the drug product it is absorbed into the surrounding tissue or the blood. The distribution of the drug throughout an organism, its metabolism and ultimate elimination, can vary significantly from person to person and from animal-to-animal. However, the general behavior of a drug *in vivo* can be characterized using statistical models. The field of pharmacokinetics encompasses the study of the kinetics of drug absorption, distribution, and elimination via metabolism and excretion. Pharmacokinetic models are used to determine the rate of drug absorption, distribution and elimination. Therefore pharmacokinetics is a crucial step in the determination of the dosage levels needed for an effective therapeutic without toxicity.

The development of pharmacokinetic profiles involves both experimental and computational approaches. The experimental part involves the collection of blood samples at defined intervals following drug administration. Urine is also often collected and analyzed to identify metabolites. To perform PK studies, it is important to have robust analytical methods that can be used to quantify the drug in plasma, serum, and urine samples. The computational approach involves modeling of the ADME process using the plasma, urine, and fecal concentrations of the drug. The biological data is graphed (e.g. plasma drug concentration vs. time of collection) and then the drugs behavior is analyzed computationally to predict the pharmacokinetic and pharmacodynamics properties of the drug.

Oral dosing is the most common and accepted route of drug administration and is normally associated with a high level of patient compliance. However, most peptides are not stable in the GI tract since it is designed for the digestion and adsorption of proteins. Its primary role is to bring nutrients and fluids into the body, while protecting against toxins, pathogens, and antigens [1]. The same processes that protect us from undesirable substances hamper the absorption of pharmaceuticals following oral administration. A physical barrier exists in the intestinal lining that is composed of: cell membranes, tight junctions between epithelial cells, a phospholipid bi-layer, mucus, efflux systems, and luminal enzymes.

Four main physical barriers have been reported as impeding the absorption of peptide-based pharmaceuticals in the GI tract [2]. These barriers are: (1) the unstirred water layer, (2) the membranes of the intestinal epithelial cells, (3) tight junctions, and (4) a mucosal layer as seen in Figure 3.1. Epithelial cells of the intestine are covered by a hydrophilic aqueous boundary consisting of water, mucus, and glycocalyx [1]. This layer is known as the unstirred water layer. The unstirred water layer has been shown to be of limited importance as a barrier in the *in vivo*

process to both actively and passively absorbed solutes [3]. However, the mucus element of this layer can restrict drugs from reaching the epithelial surface. This is due to the composition of the mucus itself. Glycoproteins (mucins) are the major component of the mucus and these can stabilize the unstirred water layer by interacting with diffusing molecules and components through steric, interactive, and dynamic properties [4,5].

The next physical barriers to be considered are the membranes of the intestinal epithelial cells and tight junctions between the cells. The intestinal epithelium is composed of columnar cells that include a combination of enterocytes, goblet cells, endocrine cells, and Paneth cells [6]. Passive diffusion, carrier-mediated transport, or vesicular transport are the only means by which a drug can be absorbed via the transcellular pathway. Lipid-soluble molecules can passively diffuse across plasma membranes. However, in order to move across cell membranes, a drug needs to have the appropriate size, charge, hydrogen bonding potential, and solution conformation [7]. These requirements are summarized in what is known as Lipinski's Rule of Five [8]. Lipinski's rule states that for a drug to have high oral bioavailability it cannot violate more than one of the following criteria: no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, a molecular mass less than 500 Daltons, and a log P less than 5. Thus molecules that are large or highly charged cannot pass through membranes unless they are transported via an active transport carrier. Since peptides typically have high molecular weight, they are hindered with regards to their passive diffusion through intercellular spaces and are almost always actively transported.

Movement of large molecules through the intercellular spaces is also prevented by tight junctions. The para-cellular pathway of drug absorption involves an aqueous extracellular route through the intercellular spaces between adjacent epithelial cells. Due to the absence of

proteolytic activity, this pathway has seen recent interest in the delivery of peptides [9]. However, hydrophobic intercellular material circumscribes each intestinal epithelial cell to form a seal that restricts diffusion of molecules according to size and charge [7,10]. These junctional complexes consist of three parts: tight junctions, adherens junctions, and spot desmosomes [11]. The tight junction is the only junction that has the potential to occlude. It controls diffusion of solutes through the para-cellular route and maintains polar distributions of the plasma membrane proteins at apical and basolateral surfaces [12,13].

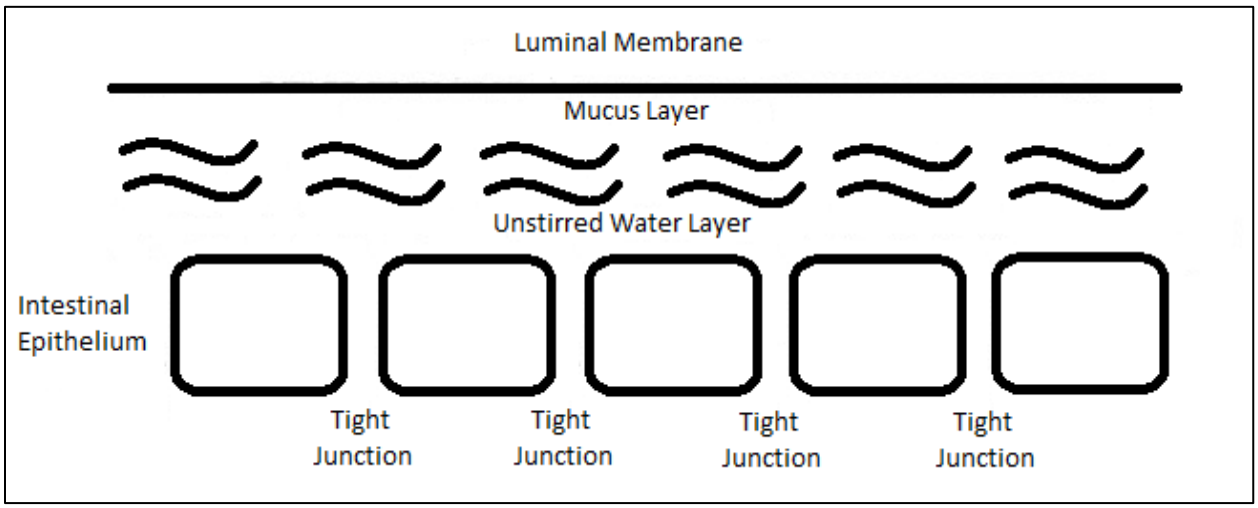

Figure 3.1 Main physical barriers of the GI tract

In addition to physical barriers, biochemical barriers exist. Degradation of peptides within the lumen of the GI tract can occur due to metabolism by digestive enzymes or luminal microorganisms. The enzymatic barrier is a critical impediment to overcome when considering the delivery of peptide pharmaceuticals. Enzymes in the GI tract catalyze digestive processes through hydrolytic cleavage of peptide bonds and chemical modifications. Proteolysis starts in the stomach in the presence of pepsin and occurs throughout the entire intestine. Enzymes released in the pancreas account for the majority of the luminal degradation of peptides, and

proteases are present in most anatomical sites, leaving peptides vulnerable to degradation throughout the body. Furthermore, instability in acidic environments is also a concern for oral peptide delivery. The pH in different parts of the GI tract varies from 1.5 to 8.5, and as a result, hydrolysis can be a concern for drugs that are pH-dependent.

Physical and biochemical barriers cannot fully explain the barrier function of intestinal mucosa. Efflux systems (e.g. P-glycoprotein) along with intracellular metabolism can significantly contribute to poor bioavailability of peptides [7]. Active efflux is a biochemical mechanism that moves compounds out of a cell. Some efflux systems are drug specific and can result in the removal of the drug, contributing to poor availability. P-glycoprotein (P-gp) is an efflux protein located in the apical domain that pumps compounds from within the cell into the intestinal lumen [14]. Many drugs have been found to be substrates, inhibitors, or inducers of P-gp [15].

3.2 Animal Models

Animal models are used in various stages of drug development to aid in the assessment of drug absorption, toxicity, exposure, and potential drug-drug interactions. *In vivo* animal studies, when done in concert with *in vitro* model systems, can be extrapolated to develop human dosing regimens for clinical testing. When considering pharmacokinetics, the animal model that is used must have similar biochemical and physiological systems governing drug adsorption, distribution, metabolism, and excretion (ADME) to humans. It is not possible to predict PK parameters of a drug from an animal model alone. However, using the correct animal model makes it possible to extrapolate dosage, duration, and toxicity into humans.

An example of an animal model that is widely used for PK studies are rodents, including rats and mice. Rodents are categorized as gastric-acid secretors and are used in the study of pH-sensitive adsorption. Intestinal permeability of humans and rodents can be correlated for drugs that passively diffuse through the enterocytes. It has been shown that a correlation of drug intestinal permeability exists between human and rat small intestine regarding both carrier mediated absorption and passive diffusion mechanisms [16]. This is due to similar gene expression of many common transporters between the human and rat duodenums. Multidrug resistance protein-3 (MDR-3) and glucose transporter 1 and 3 are similarly expressed in both human and rat intestine [16]. Additionally, rodents and humans exhibit similar expressions of metabolizing enzymes in the intestines and the liver [16]. Therefore, rodent models are suitable in the prediction of drug absorption.

Absorption enhancers are compounds that disrupt the intestinal barrier allowing a drug to penetrate the epithelial cells [17]. However, disruption of the intestinal barrier can cause tissue damage and as a result, absorption enhancers must meet specific criteria. These include: reproducible drug absorption, predictable drug permeation, low toxicity, knowledge of the mechanism of action of the absorption enhancer, and reversible/minimal tissue damage. To mitigate the biochemical barrier, enzyme inhibitors can be used. However, the use of enzyme inhibitors has several drawbacks including disturbance of the digestion of proteins, stimulation of protease secretion, and various adverse events have been documented. Therefore, the challenge with this approach has been the development of a controlled, localized delivery of both drug and inhibitor [18].

3.3 Delivery of Peptide-Based Pharmaceuticals

Some of the advantages and disadvantages of peptides as drugs, compared to small molecule drugs, are summarized in Table 3.1. Despite the disadvantages listed in the table, many peptide-based drugs have already reached the market. Analysis of the structural data for synthetic peptides [19] shows that the majority of current peptide-based synthetic products consist of 8–10 amino acids. Almost invariably, commercially available peptide therapeutics are delivered via injection or IV infusion due to the low oral bioavailability of these types of molecules. However, a major driving force for the interest in peptides and proteins as drugs is that they bind with high specificity to their *in vivo* targets, resulting in high potency of action. Peptides interact specifically with biological receptors making them potent endogenous hormones, growth factors, neurotransmitters and signaling molecules. In principle peptides could have many valuable applications in medicine but so far applications of synthetic peptides have been severely limited by their low systemic stability, high clearance, poor membrane permeability, negligible activity when administered orally, and high manufacturing costs.

Advantages	**Disadvantages**
High potency	Poor metabolic stability
High selectivity	Poor membrane permeability
Broad range of targets	High production costs
Potentially lower toxicity than small molecules	Rapid clearance
Low accumulation in tissues	Poor solubility
High chemical/biological diversity	Poor oral bioavailability

Table 3.1 Advantages and disadvantages of peptide-based pharmaceuticals

Despite their drawbacks, peptides are becoming increasingly important in the systemic treatment of certain conditions [2,20]. Most drugs with a peptidomimetic structure are difficult to

deliver orally because of poor stability in the gastrointestinal (GI) tract and low membrane permeability [21-23]. However, most patients find injections to be uncomfortable and difficult. This can lead to poor compliance regarding administration.

Strategies for overcoming both physical and biochemical barriers for administration of peptide pharmaceuticals include many different approaches: chemical modification, pro-drug delivery, targeted delivery, co-administration of enzyme inhibitors, absorption enhancers, and interfering with ATP hydrolysis [24]. However, attempts to overcome the enzymatic barrier alone have met little success [25]. This is because many factors are in play when considering the pharmacokinetics of peptide drugs. Thus the goal of peptide delivery is to overcome both the physical and biochemical barriers through chemical modifications of the peptide.

There are many strategies for delivery of peptide pharmaceuticals. These include but are not limited to: prodrug development, structural modifications, and the addition of absorption enhancers or enzyme inhibitors. A prodrug is a pharmacologically inactive derivative of a parent drug that must be transformed in the body into metabolites that have pharmacologic activity. Prodrugs are designed to overcome limitations of the parent drug. Most prodrug approaches applied to peptides have focused on modification of functional groups in the C-terminus. With cyclic prodrugs, C-terminal carboxyl and N-terminal amino groups are protected from carboxypeptidases and aminopeptidases through the formation of a ring structure [26]. Cyclic modifications also limit the amount of hydrogen bonding in aqueous environments, increasing the ability of the molecule to diffuse across the mucosa of the GI tract.

Two common approaches to chemical modification of peptides, for overcoming both physical and biochemical barriers, is the attachment of polyethylene glycol (PEG) and

lipidization conjugation. PEGylation has been shown to improve biologic and pharmacologic properties of peptides [27]. Due to the favorable characteristics of PEG, the FDA has approved PEG modified drugs for IV, oral and dermal delivery. The attachment of PEG to peptide drugs has shown to decrease enzymatic activity, immunologic response, and overall clearance [28]. Lipidization is the conjugation of a fatty acid to a peptide. This method allows for the regeneration of the active peptide in tissues and the blood through the process known as reversible aqueous lipidization (REAL) and can be delivered in aqueous solutions. REAL modified peptides have been shown to increase GI stability, epithelial absorption, and plasma half-life [29].

Subcutaneous administration of zyklophin (1-3 mg/kg) has been previously explored [30]. Following SC administration, the compound showed dose dependent antagonism of antinociception induced by the KOR agonist in U50,488 in mice as determined via a 55°C warm water tail withdrawal assay. Zyklophin had no effect on morphine mediated antinociception. This further demonstrated its high KOR selectivity. Additionally, subcutaneous administration of zyklophin prevented stress-induced reinstatement of cocaine-seeking behavior in a conditioned place preference assay [30].

3.4 Experimental

As previously described in Chapter 2, adult male CD1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in accordance to the National Institute of Health Guide for Care and Use of Laboratory Animals. Adult male CD1 mice, weighing 30-35g, were administered zyklophin by the subcutaneous route at 3 mg kg^{-1} in PBS. The dosing solution (0.100 mL) for the subcutaneous administration was geared to the

weight of the mice. The animals were sacrificed, and 1.0 mL of blood drawn at 15, 30, 60, 120, 240, 480 and 720 min was immediately centrifuged at 15,000 rpm for 15 min to harvest plasma. The samples were kept on dry ice and processed on ice. Prior to storage at -80°C samples were treated with formic acid to achieve a final formic acid concentration of one percent. Plasma samples were analyzed according to methods described in Chapter 2.

Liquid chromatography was performed using an ACE C18 column (5µm, 50 x 3.0 mm), supplied by MAC-Mod, with a flow rate of 0.2 mL/min and an injection volume of 30 µL using a Waters Acquity Classic UPLC (Waters Corp., Milford MA) coupled to a triple quadrupole mass spectrometer (Quattro Ultima Micromass Ltd. Manchester UK) operating in the positive-ion mode. The peptides were separated using the following gradient of aqueous 0.08% formic acid in water (mobile phase A) and acetonitrile containing 0.08% formic acid (mobile phase B): 0 min (1% B), 5 min (60% B), and 7 min (1% B). From time 0 min to 5 min the %B was increased with a profile curve of 9 (see Figure 2.9). From time 5 min to 7 min the %B was decreased with a profile curve of 6.

The precursor ions of zyklophin $[M+3H]^{3+}$ *m/z* 470.5 and its internal standard $[M+4H]^{4+}$ *m/z* 345.0 were first isolated and then subjected to collision-induced dissociation to give their fragment ions. Data acquisition was carried out with Mass Lynx 4.1 software with the following settings: capillary voltage, 2800 V; cone voltage, 25 V; source temperature, 100 °C; desolvation temperature, 200°C; cone gas flow, 82L/h; desolvation gas flow, 1157 L/h. Q1 and Q3 resolution were 0.8uFWHH. The argon filled collision cell pressure was 1.63×10^{-3} mbar on a gauge in-line with the cell. Multiple-reaction-monitoring was used for zyklophin $[M+3H]^{3+}$ *m/z* 470.5 $\rightarrow$ 91.2, and for the internal standard $[M+4H]^{4+}$ *m/z* 345.0 $\rightarrow$ 150.2 with a collision energy of 25 eV and dwell time of 0.3 s (see Figure 2.4).

Stock and working solutions of zyklophin and internal standard were prepared in polypropylene tubes with Milli-Q water. The calibration standards were prepared freshly in blank (drug-free) mouse plasma using the zyklophin working solution. The plasma calibration curve consisted of: zero standard, 10, 15, 25, 50, 100, 200, 500, and 1000 ng/mL. See Figure 2.5. To a mixture of 25µL of ice-cold plasma and 25µL of ice-cold internal standard (500 ng/mL prepared in ultrapure water) was added ice-cold acetonitrile (200µL) to precipitate the proteins out of solution. Following the precipitation, samples were vortexed and centrifuged at 10,000 rpm for 10 minutes. Immediately following centrifugation, 150µL of the supernatant was dried down in a speed vacuum concentrator. The residue was then reconstituted in 75µL of 0.1% formic acid and analyzed by LC-MS/MS according to the method described above.

3.5 Results and Discussion

Zyklophin was administered to 33 CD1 mice by subcutaneous injection of 3 mg kg^{-1} in PBS. The mean plasma zyklophin reached a maximum concentration at 30 min post-dose. The peptide exhibited a maximal plasma concentration of 1.8 µg/mL with a high plasma clearance (see Table 3.3). See Table 3.2 regarding specific PK parameters for subcutaneous dosing of zyklophin. These results are consistent with the desired short duration kappa opioid receptor (KOR) antagonist activity *in vivo* of zyklophin (3 mg kg^{-1} administered subcutaneously), which lasts for less than 12 hours. Therefore, the selective KOR activity and favorable pharmacokinetic properties of zyklophin make it an ideal candidate for potential drug development for drug abuse [30].

Dose	t_{max}	C_{max}	AUC	$t_{½}$
mg/kg	Hr	µg/mL	hr · µg/mL	hr
3	0.5	1.817	2.044	Unable to calculate

Table 3.2 PK parameters of Zyklophin administered via the subcutaneous route

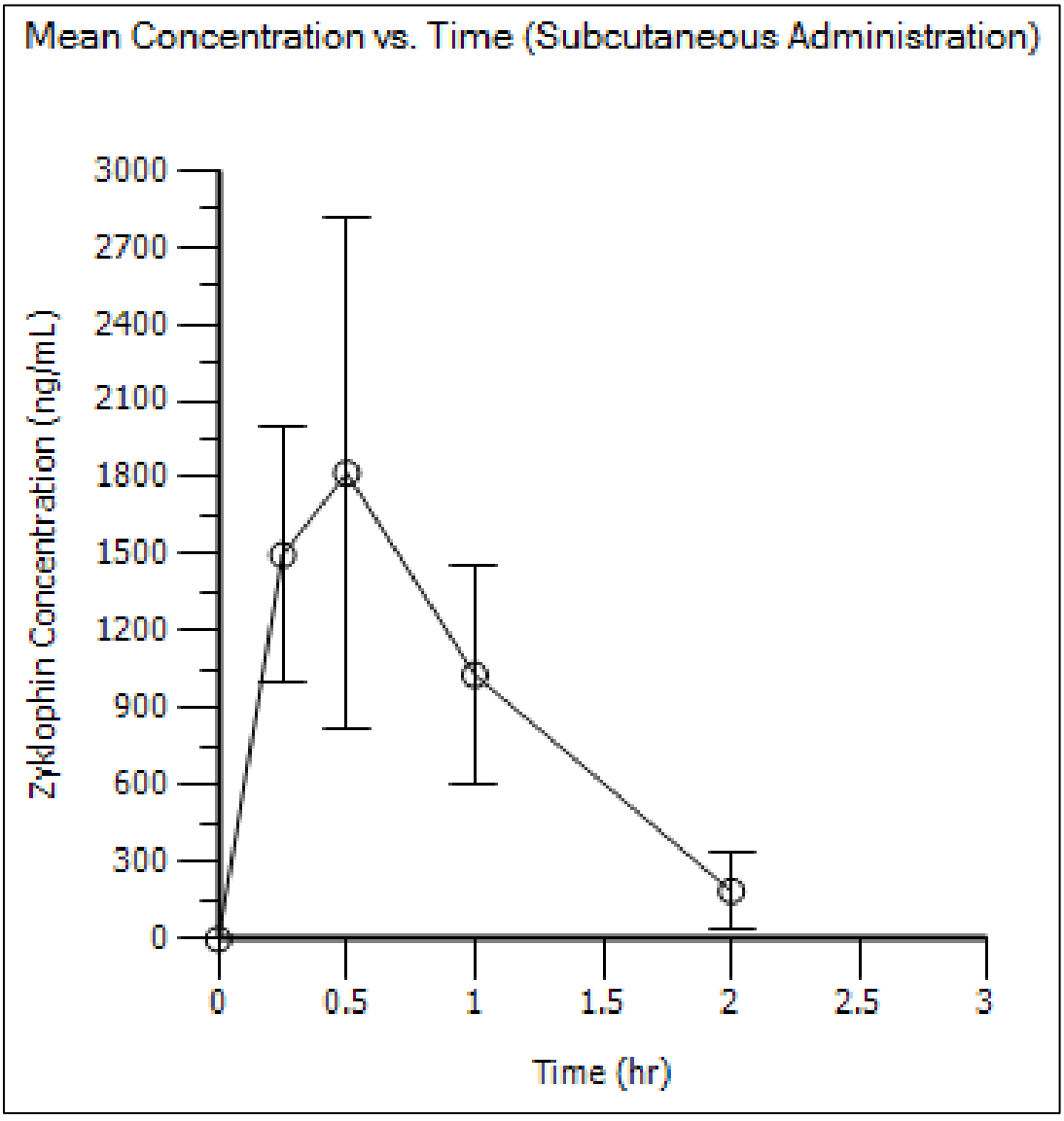

Figure 3.2 Mean concentration vs. time PK profile (mean and standard deviation, n = 5 at each time point) of zyklophin following subcutaneous administration. See Table 3.3 for individual concentration data.

Time (h)	Zyklophin ng/mL Concentration
0.25	1108
0.25	1688
0.25	1953
0.25	1916
0.25	832
0.5	2784
0.5	3024
0.5	1212
0.5	1177
0.5	894
1	1733
1	1094
1	820
1	881
1	626
2	321
2	176
2	351
2	62
2	24
4	Below LLOQ
4	Below LLOQ
4	Below LLOQ
4	Below LLOQ
4	Below LLOQ
8	Below LLOQ
8	Below LLOQ
8	Below LLOQ
8	Below LLOQ
8	Below LLOQ
12	Below LLOQ
12	Below LLOQ
12	Below LLOQ

Table 3.3 Concentration data via LC/MS-MS of plasma samples following subcutaneous administration (LLOQ 10 ng/mL)

3.6 References

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Chapter Four:

Future Directions

4.1 Thesis Summary

Peptide-based drugs have been shown to be successful pharmaceutical agents exhibiting high activity, high specificity, low toxicity, and minimal drug-drug interaction. However, peptide-based drugs have been under-utilized due to low oral bioavailability. Initial testing has shown that zyklophin crosses the blood brain barrier to antagonize the KOR in the CNS following *in vivo* administration. Zyklophin's duration of activity following a systemic dose is less than 12 hours where as a non-peptide KOR antagonist, such as nor-BNI, exhibits duration of activity up to 28 days after a single dose is administered. This prolonged activity of nor-BNI complicates its use as a pharmacological tool and as a potential therapeutic agent whereby increasing the importance of peptide-based KOR antagonist. Due to modifications of the C-terminus and address domain, zyklophin has an increased metabolic stability, high KOR selectivity, and antagonist activity.

In this thesis, the pharmacokinetics of zyklophin following subcutaneous administration were investigated using LC-MS-MS. Zkylophin exhibited a maximal plasma concentration of 1.8 µg/mL with a high plasma clearance (see Table 3.3). The subcutaneous PK results are consistent with the desired short duration kappa opioid receptor (KOR) antagonist activity *in vivo* of zyklophin (3 mg kg^{-1} administered subcutaneously), which lasts for less than 12 hours. Therefore, the selective KOR activity and favorable pharmacokinetic properties of zyklophin make it an ideal candidate for potential drug development for drug abuse.

4.2 Future Directions

Future investigations into the metabolism and distribution of zyklophin *in vivo* in biological tissues will be needed to further assess its potential as a pharmaceutical agent. Since

zyklophin shows promise as a treatment of cocaine addiction (short duration kappa opioid receptor (KOR) antagonist activity *in vivo*), the development of an appropriate formulation is necessary to ensure a high level of compliance.

Microdialysis studies can be used to examine the site specific metabolism of zyklophin. If zyklophin is delivered peripherally, transport studies can be characterized by sampling via the microdialysis probe as the release of neurotransmitters can be investigated. By utilizing radiolabeling and scintillation counting, permeation studies can be conducted to determine if other un-labeled peptides are competing for the same transport system.

Appendix 1

HLB Sample Preparation Method – Zyklophin

1. 25 µL of plasma sample (blank plasma for control blanks and zero standard)
2. 25 µL of internal standard (prepared in ultrapure water)
3. 200 µL of ultrapure water
4. Vortex and centrifuge for 10 minutes at 5000 rpm
5. Rinse an Oasis HLB 96-well Plate, 30 mg Sorbent per Well, 30 µm Particle Size with 200 µL ACN.
6. Rinse with 200 µL MeOH.
7. Wet with 200 µL ultrapure water.
8. Load 150 µL sample.
9. Wash with 200 µL ultrapure water.
10. Wash with 200 µL 0.1% formic acid.
11. Wash with 1% ACN.
12. Wash with 5% ACN.
13. Elute with 100% MeOH.
14. Dry down with sample concentrator.
15. Reconstitute residue with 75 µL 0.1% formic acid.
16. Inject onto LC-MS/MS platform.