Characterization of Serotonin and Gut Microbiota Following Exposure to Antibiotics in White-tailed Deer (*Odocoileus virginianus*)

by

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TABLE OF CONTENTS

ABSTRACT

In this thesis we will be discussing the importance of developing baseline serotonin concentrations in white-tailed deer serum and urine samples, both are minimally invasive tissues. An analytical method for the detection and confirmation of serotonin, 5-hydroxytryptamine (5-HT), in white-tailed deer tissues was developed and validated. Serum and urine samples were extracted with acetonitrile. Liquid chromatography separation was attained on a Phenomenex C_{18} column with a Security Guard ULTRA guard column with gradient elution using a mobile phase of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. This methodology was applied to baseline (control), chlortetracycline (CTC) treated, florfenicol treated and tulathromycin treated white-tailed deer serum and urine samples.

We will also be discussing the impact chlortetracycline, an antibiotic growth promoter (AGP), has on serotonin concentrations as well as on the white-tailed deer gut microbiota. Florfenicol and tulathromycin impacts on serotonin concentration changes were also investigated. Next-generation sequencing of 16S rRNA was used to characterize the bacterial communities in the rumen and fecal samples of white-tailed deer treated with chlortetracycline.

This research will aid the white-tailed deer breeders and other researchers toward the characterization of "normal" concentrations of serotonin in healthy animals. This characterization will allow for the development of a biomarker using non-invasive sample tissues in sick animals, for example, non-clinical cases of chronic wasting disease. It will also allow some further insight into whether the use of antibiotics as growth promoters (AGP), such as chlortetracycline, is affecting serotonin concentrations in white-tailed deer. If serotonin concentrations are being affected by the use of AGPs, it could indirectly affect behaviors that are associated with serotonin and overall herd health. Antibiotics inhibit the growth of microorganisms so characterizing the gut microbiota in white-tailed deer in control and chlortetracycline treated samples will also allow insight into whether the use of AGPs impact the abundance and/or diversity of bacterial communities in the gut of white-tailed deer.

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Neurodegeneration

Neurodegeneration is defined as the degeneration of the central nervous system which includes the loss of neurons in the brain and/or spinal cord [1]. This loss of neurons can cause both deficits and impaired neurotransmission [2]. A few examples of neurodegenerative diseases in humans are Alzheimer's disease (AD), Parkinson's Disease (PD), Creutzfeldt-Jakob disease (CJD), and Huntington's disease (HD). There are also neurodegenerative diseases found in animals such as bovine spongiform encephalitis (BSE), scrapie, and chronic wasting disease (CWD).

Some of these neurodegenerative diseases are classified as transmissible spongiform encephalopathy (TSE) diseases, also known as prion diseases, that are characterized by small holes in the brain that result in a "spongy" appearance [3]. Examples of TSE disease are CJD, BSE, scrapie, and CWD [4]. Prions are unusual pathogenic agents that mainly consist of a misfolded form of a naturally occurring host protein [5]. Prions are unique in that they do not have a nucleic acid genome like DNA. So unlike other pathogens, it does not need DNA or RNA to replicate [5].

Neurotransmitter changes are commonly found and are characteristics of some neurodegenerative diseases. Alzheimer's and Parkinson's disease both have characteristic neurotransmitter changes. In AD, the cholinergic and glutamatergic neurotransmission systems are affected [2]. The cholinergic system involves the loss of cholinergic neurons, decreased acetylcholinesterase activity, and abnormal cholinergic signaling in the basal forebrain [2]. It is known that the cholinergic systems are involved in both cognitive and non-cognitive behaviors that are present during the progression of AD [6]. Cognitive behavior involves attentional functioning and processing as well as memory [2]. Changes in serotonin, dopamine and γ-aminobutyric acid (GABA) are also associated with AD pathology. These changes in neurotransmitter concentrations in the central nervous system are associated with many neurological conditions [7].

The main characteristic of Parkinson's disease is a loss of dopaminergic neurons of the substantia nigra pars compacta [6]. This loss in dopaminergic neurons results in an imbalance of γ-aminobutyric acid (GABA) and glutamate neurotransmission in the nigrostriatal system [8]. There is also a decrease of serotonin in Parkinson's disease [9]. These changes in neurotransmitter concentrations are beginning to play a key role in diagnosing and monitoring neurodegenerative diseases [7] and can be adapted to further the understanding of chronic wasting disease neurodegenerative conditions in cervids.

Chronic Wasting Disease

Chronic wasting disease (CWD) is a chronic, fatal, neurodegenerative disease induced in cervids following exposure to prion proteins. The prion proteins in CWD (PrP^{CWD}) accumulate in the central nervous system and results in neurodegenerative changes [10]. CWD PrP accumulates in the retropharyngeal lymph nodes before the brain so it is a very important tissue for diagnostic testing. CWD shares similar traits with other neurodegenerative diseases such as AD and PD in terms of protein misfolding occurring and molecular pathogenesis [11]. In Alzheimer's disease, the misfolded proteins are known to be amyloid-beta proteins which form Aβ plaques and tau proteins that form neurofibrillary tangles [12, 13]. In Parkinson's disease, the misfolded protein is thought to be α-synuclein which is a key component of Lewy bodies and Lewy neurites [14].

As of January 2019, CWD has been reported in free-ranging deer in 24 states in the United States but it has also been reported in captive deer, elk, and reindeer [15]. Mule deer (*Odocoileus hemionus*), white-tailed deer (*O. virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*) and moose (*Alces alces shirasi*) are the known hosts of CWD and all members of the family Cervidae [16]. As of now, there are no known cases of CWD being transmitted to humans and causing a human prion disease [10].

The origin and mechanism of transmission of CWD are unknown. Prions do not have or need a nucleic acid genome to replicate and they are extremely resistant to

treatments that would normally destroy other infectious agents, including ultraviolet (UV) and ionizing radiation (IR), exposure to chemical disinfectants, and heat treatments. Since the CWD prion is resistant to common treatments, it is able to remain infectious in the environment for many years [5].

To date, there are very limited options for diagnosing an animal with CWD and there are no effective treatments or vaccines. A few diagnostic tests include the sampling of rectal biopsies, tonsil biopsies, retropharyngeal lymph nodes and the obex. Tonsil biopsy, retropharyngeal lymph nodes, and obex sample collections of diagnostic testing are very invasive. The tonsil biopsy can be collected from live animals but it requires the animals to be anesthetized prior to the collection of the sample. The retropharyngeal lymph nodes and obex samples are collected post-mortem. Rectal biopsies are a less invasive sample to collect since they can be collected from live, restrained animals. The gold standard tissues for postmortem diagnosis are the dorsal motor nucleus of the vagus in the obex region of the brainstem and the medial retropharyngeal lymph nodes [11]. The current diagnostic tests used for the detection of CWD PrP includes the following: Immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA) and Western blot. The preferred test is IHC and it is used to confirm positive ELISA screening tests [17].

If we could develop baseline neurotransmitter levels in white-tailed deer, we could use those levels to identify herd health and pave the way for future studies to identify neurotransmitter changes in CWD cases through minimally invasive samples, blood and urine. These neurotransmitter changes would then allow for a minimally invasive pre-screening diagnostic indicator for CWD to be developed in future studies.

Serotonin

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter. Researchers have found serotonin in numerous tissues including brain, lung, kidney, platelets, and the gastrointestinal tract [18]. Serotonin and its receptors play a key role in practically all brain functions including mood, behavior,

sleep cycles and appetite [18]. The pathogenesis of many psychiatric and neurological disorders has been associated with the dysregulation of the serotonergic system [19].

Overview of Serotonin Biology

The main serotonergic pathway begins in the raphe nuclei of the brainstem and projects throughout the brain and spinal cord [20]. The location of serotonin-containing neuronal cell bodies can be subdivided into two groups based on the distribution of the neurons and main projections. The first subgroup of the serotonergic pathway is the rostral group with major projections to the forebrain innervating the hypothalamus, the basal ganglia, amygdala, cingulum, the medial cerebral cortex and part of the hippocampus [9]. The second subgroup is the caudal group with major projections to the caudal brainstem and the spinal cord [9]. There are fourteen serotonergic receptor subtypes which are expressed throughout the central and peripheral nervous system [21]. To the best of our knowledge, the distribution and locations of serotonergic receptor subtypes has not been characterized in white-tailed deer. Serotonin does not cross the blood-brain barrier but serotonin from the CNS is able to be transported out of the CNS into the periphery [22]. Since serotonin has not been characterized in white-tailed deer, we are making assumptions based on literature that serotonin can be transported from the CNS to the PNS but not vice versa.

In the central nervous system, serotonin is synthesized and stored in presynaptic neurons such as serotonergic neurons which are neurons that produce serotonin. In the peripheral system, serotonin is primarily synthesized and stored by enterochromaffin cells which are a type of neuroendocrine cell that is located along the epithelium of the lumen of the digestive tract and platelets [18, 23]. Platelets can also synthesize serotonin but in much smaller amounts [18]. The serotonin produced from enterochromaffin cells is released into portal circulation and taken up into platelets [18]. The majority of serotonin is located in the peripheral nervous system which accounts for 90-95% of serotonin produced. About 99% of all serotonin is located intracellularly which demonstrates how tightly regulated it is [18].

Overview of Serotonin Biochemistry

Serotonin is synthesized from the amino acid precursor tryptophan which is facilitated by the transport of L-tryptophan from the blood into the brain [20, 24]. The main source of tryptophan is through dietary protein which means the entry of tryptophan into the brain is related to its concentration in blood [24]. Serotonergic neurons contain tryptophan hydroxylase which generates 5-hydroxytryptophan (5-HTP) and L-aromatic amino acid decarboxylase which generates serotonin, 5-hydroxytryptamine (5-HT) [20]. Tryptophan hydroxylase is the rate-limiting enzyme for the synthesis of serotonin and it is synthesized in serotonergic cell bodies of the raphe nuclei. Tryptophan hydroxylase is only found in cells that synthesize serotonin [24]. There are two isoforms of tryptophan hydroxylase: TPH1 which is expressed in the periphery and TPH2 which is expressed in the central nervous system. L-aromatic amino acid decarboxylase then converts 5-HTP into serotonin (5-HT) [24].

Serotonin and Associated Behavior

Serotonin is important in the regulation of mood, attention, memory, perception, anger, aggression, appetite, and sexuality as well as many others [19]. Since serotonin and its receptors play a key role in a majority of brain functions, it is hard to find a behavior that is not regulated by serotonin [19]. Each behavior is regulated by multiple serotonin receptors and each of those receptors is expressed in multiple brain regions [19].

Serotonin also regulates sleep cycles. During wakefulness, the speed at which serotonergic dorsal raphe neurons release serotonin is fast. While it is slower during non-rapid eye movement (NREM) sleep and it is the slowest during rapid eye movement (REM) sleep. This suggests that high levels of serotonin are vital for wakefulness and a reduction in serotonin levels promotes sleep [21].

Blood Serotonin

Serotonin in the peripheral system is primarily stored in platelets and it plays a key role in hemostasis. Platelets readily take up serotonin via the serotonin transporter from plasma which leaves very little serotonin circulating in plasma [18, 19].

Urinary Serotonin

Urinary serotonin is thought to represent serotonin that has not previously been in the central or peripheral nervous system. The amount of neurotransmitters from the CNS that contribute to urinary samples is unknown [25]. Serotonin found in urine is newly synthesized from serotonin amino acid precursors by the kidneys in the proximal convoluted renal tubule cells [22]. Serotonin is transported into the kidneys once it has been taken from systemic circulation and then excreted into urine [25]. Urine is a primary method of neurotransmitter elimination and it is the preferred bodily fluid for neurotransmitter measurements due to it being a non-invasive method of collection [25]. A non-invasive method is especially important for biological fluid collection in white-tailed deer.

Serotonin and Neurodegeneration

Following the observations that there is a decrease in serotonin concentrations in neurodegenerative diseases, we have selected to establish the range of serotonin in normal white-tailed deer. In AD, serotonin loss is greater and more widespread than that of other neurotransmitters in AD [26]. In PD, there is up to 50% of serotonin neurons lost in the brains of AD patients [27]. The deficit of serotonin also involves a wide range of behaviors and functions, some of which are related to symptoms of these neurodegenerative diseases.

Serotonin is known to reduce the amyloid plaque load and the activation of serotonin receptors enhances neuronal survival, decreases Aβ production, and brain Aβ levels [7]. AD patients have an accumulation of amyloid-beta plaques in the brain. Since there is a reduction of serotonin in AD, it is reasonable to assume there is no longer a reduction in the amyloid plaque load and the deactivation of serotonin receptors can lead to an increase in Aβ production, brain Aβ levels and decrease neuronal survival.

Degeneration of serotonin systems has been demonstrated by neuropathological and neuroimaging systems in Alzheimer's disease [26]. There are reduced serotonin receptor levels in AD patients. The serotonergic deficit in AD is

associated with numerous symptoms, including depressed mood, anxiety, impulsivity, agitation, restlessness and aggressiveness [28, 29]. Reduced cortical availability of serotonin due to the degeneration of the raphe nucleus is associated with Parkinson's disease [30]. There is a loss of serotonin in the striatal and extra-striatal serotonin markers during the course of PD [9]. These abnormal serotonergic neurotransmissions may contribute to the motor and non-motor characteristics associated with PD, including dyskinesias, tremor, depression, apathy, anxiety, weight loss, fatigue, and visual hallucinations [9, 27].

Antibiotics: Gut Microflora and Neurotransmitters

Chlortetracycline

The public health concerns regarding the quality and safety of food from food-producing animals have increased in recent years due to problems associated with the use of antibiotics as growth promoters (AGP). AGP is defined as antibiotics administered to animal feed at sub-therapeutic levels to enhance growth and improve feed efficiency [31]. Numerous antibiotics have been used as growth promotors such as penicillin, tetracyclines, aminoglycosides, sulfonamides, and chloramphenicol [32]. The mechanism of action of orally ingested AGP is primarily focused on the interactions between the antibiotic and the intestinal gut microbiota that could lead to the reduction of the bacterial community [31]. As a result, the Food and Drug Administration (FDA) has established a veterinary feed directive recommending limitations on the use of antibiotics in animal husbandry [33]. The use of antibiotics as a growth promotor is practice by deer breeders as a part of their health and husbandry program.

Chlortetracycline (CTC) is a naturally occurring tetracycline identified from the fungus *Streptomyces aureofaciens* in 1948 [34]. CTC is approved for use in swine, poultry, cattle, and sheep for treating respiratory and systemic infections [35]. CTC has been used as an AGP to aid in the productivity of livestock production and to improve the health and well-being of animals [31]. It is active against a wide range of gram-positive and gram-negative bacteria. The CTC mode of action works by binding

reversibly to the 30S ribosomal subunit which blocks the aminoacyl-tRNA from the acceptor site on the mRNA-ribosomal complex. This inhibits protein synthesis [1, 2].

Like other ruminants, the white-tailed deer gut microbiome is characterized by its diversity, high density, and complex interaction. It is composed of diverse and complex microbial communities such as archaea, bacteria, protozoa, fungi, and bacteriophages [36]. The health and production of ruminants is highly dependent on the rumen microbial community, that is highly responsive to dietary changes [37, 38]. Thus, the diet structure is the most significant driver of the hierarchical structural changes in bacterial communities in healthy growing ruminants [39]. The gut microbial community supports the host in the extraction of nutrients, immune system and epithelium growth, and protects the host against pathogens [36].

Antibiotics added to feed presents a selective pressure that may lead to permanent changes in livestock commensal microorganisms. The literature focuses primarily on domesticated sheep [40-42], bovine [37, 38], goats [43-45], and swine [46]. There are also several studies on non-domesticated animals (cervids) such as moose [47, 48], reindeer [49], and white-tailed deer [50]. To the extent of the literature, this study will be the first on the changes of antibiotics on serum and urine serotonin concentrations and the microbiome of white-tailed deer.

Bacteria

Microbiota, or microflora, is defined as the totality of microorganisms in a specific habitat [51-53]. Whereas microbiome is defined as the collective genomes of all the microorganisms in a microbiota [51]. The gut microbiome and what it is comprised of is established during the host's early development and can change throughout the host's lifetime [54]. There are about 100 trillion microorganisms living in the gastrointestinal tract of humans [54, 55]. It has been thought that gut microbes were primarily commensal organisms that were only beneficial to regulating the population of pathogenic bacteria but we now know that they are symbiotes that play critical roles in aiding digestion, developing the immune system, and producing essential metabolites for the host [54, 56].

In humans, there are bacteria that are classified as potentially beneficial bacteria. Some of those are *Bifidobacterium*, *Lactobacillus*, *Eubacterium*, *Fusobacterium*, and *Campylobacter jejuni* [51, 54]. Some of the potentially harmful bacteria are Clostridia, *Staphylococcus*, *Proteus*, *Pseudomonas aeruginosa* [54]. There are bacteria that are neither defined as potentially beneficial or harmful and they are Firmicutes, *Bacteroides*, *Escherichia coli*, *Enterobacteriaceae*, and *Veillonella* [54]. There are also bacterial species that are relevant to research on Parkinson's and Alzheimer's disease. The bacteria relevant to Parkinson's disease are *Prevotellaceae* and *Enterobacteriaceae* [54]. The bacteria relevant to Alzheimer's disease are Clostridia, Bacteriodetes, and Verrucomicrobia [54]. Knowing which bacterial species are seen in normal, healthy hosts and diseased hosts are important to host health. Of course, the gut microbiome will vary from species to species. For example, in the human gut, *Prevotella* and *Ruminococcus* are dominant but in the mouse gut, they are under-represented [54].

Diet plays a large role in the gut microbiome. For example, diets that are high in animal fat and protein are associated with a wealth of *Bacteroides* and high-fiber diets with *Prevotella* [54, 57, 58]. Whereas, plant-based diets have an abundance of Bacteroidetes and Firmicutes [54, 57, 58]. Other factors affecting the gut microbiome composition include genetics, environmental influence, host health and immune function, and neurotransmitters and metabolites secreted by the host [54]. This shows that there are numerous variables to consider when looking at the gut microbiome of a species. Another example of variables impacting the gut microbiome is that in humans, there is a difference in the abundance of certain microbes depending on whether an infant is breastfed and formula-fed infants [59]. This may be a factor to consider for white-tailed deer as well since there are some white-tailed deer breeders that bottle feed fawns milk replacers (formula). There is also some distinct variability of the core microbiota between younger adults and those that are elderly [59]. So, age is going to affect the gut microbes as well.

Antibiotics and Neurotransmitters

A few studies have investigated the effect of antibiotics on neurotransmitters [60-62]. A study by Sullivan administered neomycin, streptomycin, chloramphenicol, chlortetracycline, and oxytetracycline to mice and rats twice daily [61]. The antibiotics were administered orally by stomach tube and by subcutaneous injection. The control mice and rats were given the same volume of saline. After subcutaneous injection of the antibiotics, the results for the streptomycin and chlortetracycline combination given orally showed there was a significant increase of intestinal serotonin in treated mice but no significant difference between treated groups and controls groups [61]. The reported results for neomycin was an increase in intestinal serotonin as well as an increase in blood platelet serotonin following treatment. Brain serotonin was not affected in this experiment [61].

A study by Sullivan also looked at the effects of antibiotics on bacterial flora in orally treated mice. It was determined that neomycin sterilized the bacterial flora in mice. While the streptomycin and chlortetracycline combination sterilized or partially suppressed the bacterial flora. It was also observed that the chlortetracycline alone lead to partially suppressed and altered bacterial flora. These three treatment groups also lead to a significant change in the total content in intestinal serotonin [61]. It has been suggested that the rise in intestinal serotonin was brought on by the effects produced by the antibiotics on intestinal bacteria [61]. The authors hypothesized that the sterilization of bacterial flora in the gut following exposure to the combination of streptomycin and chlortetracycline may have increased the amount of tryptophan available for serotonin synthesis by preventing the bacterial metabolism of dietary tryptophan [61].

Another study by Ge et al., administered broad-spectrum antibiotics (ampicillin, neomycin sulfate metronidazole, and vancomycin) to ten mice in drinking water for 4 weeks [60]. It was determined that there was a depletion in microbiota by the antibiotic combination and this led to a deficit of colonic serotonin in mice. There was also a decreased expression of tryptophan hydroxylase 1 (TPH1) expression in those antibiotic-treated mice [60]. While colonic serotonin decreased in the

antibiotics-treated mice, the significances of serotonin regulation by bacteria in the intestine on the concentration of serotonin in the brain and host behavior is unknown [63]. While these studies were conducted in monogastric mice, it is a good model to help with understanding the data collected from our studies. To the best of our knowledge, there are no studies that look at serotonin concentration changes following exposure to chlortetracycline or any other antibiotic in white-tailed deer.

Serotonin and the Gut Microbiome

Serotonin is an important gastrointestinal neuroendocrine factor and it is primarily synthesized by enterochromaffin cells [60]. Tryptophan, the amino acid precursor to serotonin, is found in the intestinal tract. Tryptophan in the peripheral nervous system can cross the blood-brain barrier (BBB) and participate in serotonin synthesis [63]. It is unknown how serotonin regulation by bacteria in the gut impacts the concentration of serotonin in the brain and on behavior [63]. It may be possible that microbiota can directly signal neurotransmitter-producing cells such as enterochromaffin cells that produce serotonin. This may influence neurotransmitter concentrations.

Gut bacteria are also able to produce small molecules such as serotonin, dopamine, norepinephrine, epinephrine, GABA, and acetylcholine [63]. It is known that neurotransmitters are found in the gastrointestinal tract at levels equal to or higher than those in the brain. The peripheral levels of numerous neurotransmitters are greater in the gut than the brain [63]. Indigenous microbiota plays a role in regulating the brain serotonergic system as it modulates hippocampal levels of serotonin [62, 64].

The gut-brain-axis integrates all afferent and efferent neural, endocrine, nutrient, and immunological signals between the CNS and the gastrointestinal system [51, 65]. Signaling between the gastrointestinal tract and the central nervous system is bidirectional and occurs through spinal afferents and the vagus nerve through neurotransmitters like serotonin, a few other neurotransmitters and some neuropeptides [54]. Serotonin is not only an important brain neurotransmitter, but it is also a regulatory factor in the gastrointestinal tract as well as other organ systems.

The microbiome can alter neurological function directly by controlling the concentrations of neurotransmitters in the brain and the periphery. One specific example of this direct mechanism is a decrease in peripheral serotonin serum levels in the absence of gut microbiota. This decrease in serotonin serum levels is due to lower levels of serotonin metabolites and precursors in the intestinal contents and urine [63, 66, 67]. Tryptophan could possibly cross through the intestinal barrier and the blood-brain barrier and impact the central nervous system and systemic serotonin concentrations [63].

Yano et al., compared peripheral serotonin in mice with normal populations of gut microbes with germ-free mice and found that enterochromaffin cells from germ-free mice produced less serum and colonic serotonin than those mice with normal populations of gut microbes [62]. The Yano et al., study determined that the gut microbiota regulates high levels of peripheral serotonin [62]. The gut microbiota regulates approximately 64% of colonic serotonin concentrations and 49% of serum concentrations which further supports the view that microbiota regulates serotonin metabolism mainly by affecting host colonic enterochromaffin cells [62, 68, 69]. They also noticed that serotonin levels in the germ-free mice increased when they were recolonized with normal gut microbes which means the decrease in serotonin can be reversed [62]. There was also a decreased expression of TPH1 in germ-free mice colons.

The study by Yano et al., set out to determine if specific species of bacteria are interacting with enterochromaffin cells to produce serotonin. They found that species of spore-forming bacteria increases colon and blood serotonin levels in germ-free mice [62]. Indigenous spore-forming microbes from mouse and human microbiota such as clostridiums raise certain microbial metabolites such as short-chain fatty acids. Short-chain fatty acids seem to directly signal to colonic enterochromaffin cells to stimulate serotonin biosynthesis by raising TRH1 expression in colonic enterochromaffin cells [62]. This research suggests that the gut microbiota regulates host peripheral serotonin levels. Following bacterial colonization, increased production of neurotransmitters such as serotonin (5-HT) and γ-aminobutyric acid (GABA) have been seen in the gut

[54]. These physiological changes are important to gut homeostasis and to the programming of the hypothalamic-pituitary-adrenal axis, which plays a key role in stress responses [54].

In male germ-free mice, an increase in hippocampal serotonin concentrations along with an increase in serotonin turnover in the striatum of germ-free mice has been reported [59, 70]. Sarrias et al., also reported an increase in plasma levels of both tryptophan and serotonin in germ-free mice compared to conventional animals [59, 71]. While Crumeyrolle-Arias et al., noted that germ-free rats have decreased hippocampal concentrations of serotonin which is the opposite of what is observed in germ-free mice [59, 72]. It appears that there is a species difference of hippocampal serotonin concentrations between germ-free mice and rats.

Gut Microbiome and Neurodegenerative Diseases

Microbial dysbiosis, or microbial imbalance, has been linked to neurological disorders like Alzheimer's disease and Parkinson's disease [54]. Neurodegenerative diseases are often co-morbid with gastrointestinal pathology [63, 73]. Gut microbiota are important elements in shaping the host immune system. This may influence host behavior and indirectly have effects on neurodegeneration [63]. Gut microbes influence the production of neurotransmitters such as serotonin. The central nervous system is directly connected to the enteric nervous system through the vagus nerve which provides a neurochemical pathway for microbial-promoted signaling in the gut to be sent to the brain [63, 74].

Research shows that GI dysregulation is seen several years before PD is clinically displayed. It has been hypothesized that the α-synuclein associated with PD may be transported from the gut to the brain through the gut-brain axis via the vagus nerve and spinal cord [54, 75]. If GI dysregulation is seen, it may be possible that serotonin levels are also affected since serotonin is synthesized from tryptophan in the GI tract. In post-mortem cases of early PD, Lewy bodies ("aggregated proteins, mainly alpha-synuclein and ubiquitin") were located in the enteric nervous system [54, 75]. A study showed that alpha-synuclein migrated to the brain stem via the vagus nerve

when injected in the gut walls of rats at an estimated rate of 5-10 mm/day [54, 76]. Other research has shown that dysregulation of serotonergic routes of tryptophan metabolism influences the central nervous system (CNS) pathological conditions of dementia, Huntington's disease and Alzheimer's disease [51, 77].

The Rumen

The rumen is a large chamber where the consumed feed is first exposed to microbial digestion [36]. A ruminant possesses a four-chambered stomach which consists of the rumen, reticulum, omasum, and abomasum. The rumen and reticulum contain microorganisms which breakdown their fibrous diet and provide nutrients for the host [47]. The conditions of the rumen are ideal for microbial survival and growth because the temperature remains around 36-40°C, drinking water provides a moist environment for microbial growth, ingested food delivers energy and nutrients that microbes need for growth and activity, and it is an anaerobic environment [36]. The microbes provide energy, protein, and vitamins to the host so the microorganisms in the rumen and the host have a symbiotic relationship [36].

The rumen microbial population has microbes of all three domains, Eubacteria (Bacteria), Archaea (Methanogens), and Eukarya (Protozoa and Fungi) [36]. Bacteria makes up between 40 and 90% of total microbial mass while Methanogens only make up between 2 and 4%. Protozoa between 0 and 60% and Fungi about 10% of the total microbial mass [36].

Common Methanogens in the rumen are: *Methanobacterium formicium*, *Methanobacterium bryantii*, *Methanobrevibacter olleyae*, *Methanobrevibacter millerae*, *Methanobrevibacter ruminantium*, *Methanomicrobium mobile*, *Methanoculleues olentangyi*, and *Methanosarcina barkeri*. But the most prevalent is *Methanobrevibacter ruminantium* [36]. The most prevalent bacterial species in the rumen are *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* [36].

Many studies focus on domesticated ruminants such as cattle, sheep, and goats but the diet of domesticated ruminants such as the cervids used in this study differs

from that of wild ruminants. Domesticated ruminants usually have a diet that consists of high-quality forages or concentrates and hays or silages. In contrast, wild ruminants have a diet that depends on the available browse and forages in the environment at that time [50]. It has been determined that the dominant bacterial phyla in wild Canadian cervids are Bacteroidetes and Firmicutes but the relative abundance of each varies among ruminant species and also between phases of rumen digesta such as solid and liquid digesta [50]. It is thought that the distribution of Fibrobacter is rather variable in the rumen environment and that the Fibrobacter phyla only plays an important role in fiber digestion in some ruminant species [50]. Gruninger et al., determined that Firmicutes were more prevalent in solid rumen elk samples and Bacteroidetes was found at a high prevalence in the liquid samples [50].

A study looked at the bacterial gut microbiome of free-range North American moose and found that the dominant phyla in the rumen were Bacteroidetes (27%), Proteobacteria (19%), Chloroflexi (11%), and Unclassified (11%) [48]. Another study looked at bacterial communities in moose from Vermont, Alaska, and Norway. They found that the Bacteroidetes phylum was most abundant in moose from Alaska and Norway, but the Firmicutes phylum was more abundant in moose from Vermont. In the Alaska samples, Firmicutes was the third most abundant phylum and in the Norway samples, it was the second most abundant phylum. It seemed location was a strong influence in determining the gut microbiome than either age or weight, but gender did not seem to be a strong factor [47]. Other studies found Bacteroidetes was the dominant phyla in ruminants on a high starch diet whereas Firmicutes were dominant in ruminants on a high fiber diet [47, 78, 79].

CHAPTER 2

DETERMINATION OF SEROTONIN IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) SERUM AND URINE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS AS A PRECLINICAL BIOMARKER FOR CHRONIC WASTING DISEASE

Abstract

In our laboratory, a method for the detection and confirmation of serotonin, 5-hydroxytryptamine (5-HT), in white-tailed tissues was developed and validated. Serum and urine samples were extracted with acetonitrile. Liquid chromatography separation was attained on a Phenomenex C_{18} column with a Security Guard ULTRA guard column with gradient elution using a mobile phase of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 250 µL/minute. Analysis was carried out using liquid chromatography heated electrospray ionization tandem mass spectrometry in the selected reaction monitoring interface. During method development and validation, the use of a hydrophilic interaction liquid chromatography (HILIC) column was determined to be inadequate for the determination of serotonin in white-tailed deer tissues. Serotonin concentrations obtained with the use of the HILIC column lead to significantly higher concentrations compared to the C_{18} column. We determined that serotonin concentrations using the HILIC column were inaccurate due to an unestablished chemical characteristic of serotonin or the interaction of serotonin and the column. The following data were obtained with the use of the C_{18} column. Mean recovery values ranged from 111% to 115% in serum and 112% to 113% in urine. The limits of detection for serum and urine were 1.88 ng/mL and 1.76 ng/mL respectively. The limits of quantification were 6.27 ng/mL for serum and 5.86 ng/mL for urine. Serotonin concentration levels ranged from 3.2 ng/mL to 43.15 ng/mL in serum and from 2.01 ng/mL to 20.29 ng/mL in urine. In summary, we developed a method for the determination of serotonin in white-tailed deer tissues using a C_{18} column and a heated electrospray ionization (HESI) probe. This has allowed us to develop baseline, “normal”, levels of the monoamine neurotransmitter, serotonin in white-tailed deer.

Introduction

Neurotransmitter changes are commonly found and are characteristics of some neurodegenerative diseases. Chronic wasting disease (CWD) is a chronic, fatal, neurodegenerative disease in cervids. The prion proteins in CWD (PrP^{CWD}) accumulate in the central nervous system and results in neurodegenerative changes [10]. Chronic wasting disease (CWD) has become an increasing concern for white-tailed deer breeders over the past several years because there are so many unknowns with this disease. Having an infectious agent such as a prion present in an environment could potentially cause long-term risks to cervids and ecological health. Alzheimer's and Parkinson's disease both have characteristic neurotransmitter changes. In AD, serotonin loss is greater and more widespread than that of other neurotransmitters in AD [26]. In PD, there is up to 50% of serotonin neurons lost in the brains of AD patients [27]. The deficit of serotonin also involves a wide range of behaviors and functions some of which are related to symptoms of neurodegenerative diseases. Neurotransmitters such as serotonin are used as clinically relevant biomarkers for Alzheimer's disease [7]. However, a major limitation of urinary neurotransmitter measurements is that neurotransmitters, such as serotonin, are not diagnostic for these neurodegenerative diseases but they are used as functional assessments [7, 25].

Researchers have found serotonin in numerous tissues including brain, lung, kidney, platelets, and the gastrointestinal tract [18]. Through the use of liquid chromatography-mass spectrometry, we were able to determine serotonin concentrations in serum and urine, two very non-invasive sample collections. This has been done in other studies that looked at serotonin in urine samples [22, 25, 80, 81]. The purpose of this study was to develop baseline levels of serotonin in white-tailed deer. This "normal" range will allow future studies to characterize serotonin changes in clinical and non-clinical chronic wasting disease cases similar to the approach used for AD and PD. This will eventually allow for the development of a relevant biomarker for CWD and hopefully a way to detect when the disease starts to progress.

Materials and Methods

All reagents were LC-MS grade unless stated otherwise. Methanol, water, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium formate, acetic acid (≥ 99.7) and formic acid (98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Whatman mini-uniprep syringeless filter devices, microcentrifuge tubes (1.5 mL), and disposable borosilicate glass tubes with a plain end (16x100mm) were also purchased from Fisher Scientific (Pittsburgh, PA, USA).

Standard Preparation

Working standard solutions of steroid internal standard (I.S.) were prepared from 100 µg/mL of D_3-Testosterone as stock standard (Cerilliant, Round Rock, TX, USA). We prepared 10 µg/mL as an intermediate standard and 1 µg/mL as a working solution concentration. The diluent solvent was methanol in all cases.

Animal Care and Treatment

This experiment was performed in compliance with the appropriate laws and institutional guidelines approved by the Texas Tech Institutional Animal Care and Use Committee, protocol number 14039-08.

Samples were collected from thirteen healthy white-tailed does with ages ranging from 1 to 6 years old and weights ranging from 32.70 kg to 54.80 kg. Samples were also collected from eleven healthy weight-tailed bucks with ages ranging from 1 to 4 years old and weights ranging from 82.00 kg to 143.00 kg. The white-tailed deer were kept in high fenced pens. They were given *ad libitum* access to feed and water throughout the experiment. Two white-tailed doe data were collected from a previous study where only serum was collected, so there is not a urine data match for those two animals.

All white-tailed bucks were sedated with Medetomidine HCl/Ketamine HCl at a dose of 2.5 mg/kg (1.1 mL/100 lb.) of body weight via darting. We reversed the medetomidine HCl and ketamine HCl combination with an intramuscular (IM) injection of atipamezole HCl with a hand syringe at a dose of 1 mL/100 lb. of body weight. During a previous study blood and urine samples were collected for neurotransmitter analysis from each animal on the day of euthanasia [35]. Laboratory

personnel, as well as the university's veterinarians, monitored the health of the animals. The samples were stored at –80°C until analysis.

Serum Sample Preparation

Serum preparation involved centrifuging the serum separator tube (SST) with the blood sample at 2000 rpm for 15 minutes. Serum was removed using transfer pipettes and placed in Eppendorf tubes. The serum samples were then stored at -80°C until analysis.

Serotonin determination in serum was performed using a modified version of Dong et al., [81] techniques. An aliquot of 50 μL serum sample was put into a microcentrifuge tube. An aliquot of 5 μL steroid internal standard (I.S. (1μg/mL in methanol) and 195 μL ice-cold acetonitrile were added to precipitate protein and other co-extractives. The mixture was vortexed for 30 seconds and then centrifuged at 10,000 rpm for 10 minutes at 4°C. An aliquot of 150 μL of the supernatant was transferred to a syringeless filter device and diluted with 150 μL water.

Urine Sample Preparation

Urine preparation involved taking 1 mL of urine and centrifuging it at 10,000 rpm for 10 minutes at 4°C. Steroid and neurotransmitter determination in urine was performed using a modified version Zhai et al., techniques [82]. An aliquot of 5 μL of 1μg/mL I.S. of steroids was added to an aliquot of 200 μL urine supernatant in a microfuge tube. The mixture was vortexed for 30 seconds and 195 μL ice-cold acetonitrile was added to precipitate protein and other co-extractives. This mixture was vortexed for 2 minutes and centrifuged at 5,200 rpm for 10 minutes at 4°C. A 200 μL aliquot of the supernatant was transferred to a disposable borosilicate glass tube (50 mL) and evaporated to dryness on a water bath at 35°C under a protective atmosphere of nitrogen. The residue was reconstituted with 100 μL 0.1% aqueous acetic acid-methanol mixture (90:10 v/v).

Chromatographic Conditions

Conditions for white-tailed deer serum and urine samples were optimized using a heated electrospray ionization (HESI) probe for the detection of serotonin. Chromatographic separation was carried out using a refrigerated auto-sampler (CTC PAL) system with a Phenomenex Nx 3µ particle size; C_{18} column (150mm length x ~2mm) with a Security Guard ULTRA guard column (Phenomenex, USA) as well as HILIC x 100mm length x 2.1 mm, 3 µ particle size (SEPAX, Newark, DE) on column heater coupled to a Quantum Access MAX triple stage quadrupole (TSQ) mass spectrometer (Thermo Scientific) for optimization and validation.

The column temperature was set to 30°C. Solvents that constituted the mobile phase for the C_{18} column were water with 0.1% formic acid (A), acetonitrile with 0.1% formic acid (B). Mobile phase composition (A:B; v/v) was 90:10 at 0 min, 90:10 at 3 min, 10:90 at 6 min, 10:90 at 9 min, 90:10 at 12 min, and 90:10 at 15 min with a flow rate of 0.25 mL/min and an injection volume of 20 µL. Solvents that constituted the mobile phase for the HILIC column were water with 5mM ammonium formate (A), acetonitrile with 5mM ammonium formate (C). Mobile phase composition (A:C; v/v) was 10:90 at 0 min, 10:90 at 2.5 min, 25:75 at 4 min, 25:75 at 6 min, 10:90 at 8.5 min, and 10:90 at 10 min with a flow rate of 0.25 mL/min and an injection volume of 10 µL.

LC-MS/MS Analysis

MS/MS conditions for analysis of serotonin and the internal standard [D_3-testosterone] were optimized using a HESI probe in positive polarity. A 5µg/mL standard was used to determine the optimum counts per seconds (cps) in MS and MS/MS scans. MS parameters for both the C_{18} and HILIC column are outlined in Table 2.1. Compound-specific mass spectrometer parent and product ion settings are presented in Table 2.2. White-tailed deer sample validation analyses were carried out for the HESI probe. The comparative results for these techniques were documented and discussed below.

Table 2.1 Mass Spectrometer Parameters for HESI Probe Using C_{18} and HILIC Columns

MS Parameters	**HESI**	**HESI**
Column Type	C_{18}	HILIC
Polarity	Positive	Positive
Vaporizer Temperature (°C)	317	290
Capillary Temperature (°C)	333	350
Sheath Gas Pressure [Arb], Nitrogen	40	38
Auxiliary Gas Pressure [Arb]. Nitrogen	12	7
Peak Width Q1, Q3 (FWHM)	0.70	0.70
Collision Gas Pressure (mTorr), Argon	1.5	1.5
Spray Voltage	3500	3500
Scan Method	SRM	SRM

Table 2.2 Analyte Specific Mass Spectrometer Settings Used for Serotonin Methods

Analyte	Parent Ion (Q1)	Product Ion (Q3)	Collision Energy (v)	Tube Lens (v)	Ions
Serotonin	177.109	115.058	27.848	68.225	Quantifier
		160.058	10.253	68.225	Qualifier
D_3-Testosterone	292.27	109.151	21.629	119.191	Quantifier
		97.222	19.607	119.191	Qualifier

Method Validation

The LC-MS/MS method was validated for control white-tailed deer serum and urine samples. The parameters determined were specificity, selectivity, linearity, system precision, repeatability, matrix effect, and internal quality control.

Method-Specificity/Selectivity

To determine the selectivity and specificity of the method and to demonstrate the absence of interference with the elution of the analyte, we analyzed a solvent blank followed by blank white-tailed deer serum and urine extracts. This was followed by analysis of a reference solvent standard and analysis of white-tailed deer serum and urine matrix standards. The blank white-tailed deer matrix sample was spiked with a low level, 10 ng/mL, of serotonin prior to processing and analysis.

Method-Linearity/Limit of Detection/Limit of Quantification/Precision/Range/Carryover

White-tailed deer serum and urine were used for matrix-fortified calibration curves. To our knowledge, there are no established neurotransmitter concentration levels in white-tailed deer serum and urine. The selected concentration ranges for the solvent and matrix-matched calibration standards for serotonin were: 1, 5, 10, 25, 50, 100, and 250 ng/mL (ppb). The limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated based on the blank determination using the formula $LOD = X_{b1} + 3S_{b1}$ and $LOQ = X_{b1} + 10S_{b1}$ where X_{b1} is the mean

concentration of the blank and S_{b1} is the standard deviation of the blank [83]. The LOD was set based on the measurement of seven replicates of blank white-tailed deer serum and urine and spiked with 10 ng/mL. The standard deviation from the spiked samples were multiplied by 3 (3 x SD) to establish the LOD used for this study. The standard deviation of the spiked samples was multiplied by ten (10 x SD) to establish the LOQ for this study.

Method-Recovery/Repeatability/Matrix Effects

Two concentrations of serotonin were used to establish recovery and repeatability of the current method. Spiked white-tailed deer serum and urine samples were used to determine method recovery. For this determination, untreated white-tailed deer serum and urine samples were fortified with 10 and 100 ng/mL of serotonin. This concentration was replicated seven times. The samples were analyzed for the concentration of serotonin. Blank white-tailed deer serum and urine sample extracts were used as a diluent to estimate matrix effects. To determine the matrix effects, the mean area counts of the serotonin in the spiked samples were divided by the mean area counts of serotonin standard solutions and multiplied by 100.

Method-Internal Quality Control

The internal quality control of the system was performed by injecting 25 ng/mL standard as an initial calibration verification (ICV). After every tenth sample injection in the sequence a continuum calibration verification (CCV) serotonin standard of 25 ng/mL was injected and analyzed.

Statistical Analysis

Statistical differences between means were evaluated with analysis of variance (ANOVA) test and with two-tailed Student's t-test using JMP statistical software. P values less than 0.05 were taken to be statistically significant. If there was a significant difference after the ANOVA test, a Tukey HSD test was conducted to tell where the significant difference was.

Results and Discussion

In an effort to analyze serotonin levels in white-tailed deer tissues, we utilized liquid-liquid extraction and liquid chromatographic separation followed by MS product and parent ion pattern for qualitative and quantitative determination of serotonin concentrations in blood and urine. To our knowledge, there is no data available characterizing serotonin levels in white-tailed deer tissues. This optimization was carried out using a HESI probe. There are published reports for the determination of serotonin using a C_{18} column [84-86] as well as with a HILIC column [87, 88]. All of these published reports used electrospray ionization (ESI) probes for the determination of serotonin concentrations in tissues.

In this study, the data revealed that the HILIC column did not accurately analyze serotonin levels in white-tailed deer serum or urine. Kim et al., noted that a HILIC column can accurately separate hydrophilic chemicals [86]. The HILIC column in this study did not separate serotonin clearly. It appears that the HILIC column resulted in false positive results due to an unestablished chemical characteristic of serotonin or the interaction of serotonin and the column. Another study also noted that the serotonin peak could not be separated when using the HILIC column compared to the C_{18} column [84]. Based on our current protocol and our derived data, we can confidently suggest the use of the HESI probe with the C_{18} column for the quantitative and qualitative determination of serotonin in white-tailed deer serum and urine samples.

Method Validation

We optimized chromatographic conditions for qualitative and quantitative determination of serotonin in white-tailed deer serum and urine samples. It has been reported that serotonin can be successfully separated using different chromatographic columns including the use of C_{18} columns [84-86], HILIC columns [87, 88], and Acquity UPLC BEH amide columns [88]. In this study, we used a C_{18} column (150mm length x ~2mm) with a Security Guard ULTRA guard column (Phenomenex, USA) for chromatographic separation and found no concerns with the white-tailed

deer tissue samples during separation. The HILIC column data was determined to be inaccurate based on method validation and optimization.

There are published mobile phase protocols that have been used with serotonin such as water/acetonitrile with 0.1% aqueous acetic-acid [80], water/acetonitrile with 0.1% formic acid [84], or water/acetonitrile with 25mM ammonium formate and formic acid (0.01% v/v) [88]. In this study, the mobile phase of 0.1% formic acid in water and acetonitrile provided clear chromatographic separation of serotonin in serum and urine samples. The combination of the mobile phase and the chromatographic conditions described above for serotonin facilitated the retention time of 1.08 minutes in serum and 1.21 minutes in urine. Selectivity was verified by analyzing seven replications of blank white-tailed deer serum and urine samples and it was determined that there were no internal interfering substances from the samples at the same m/z ratio of serotonin retention times Figures 2.1-4.

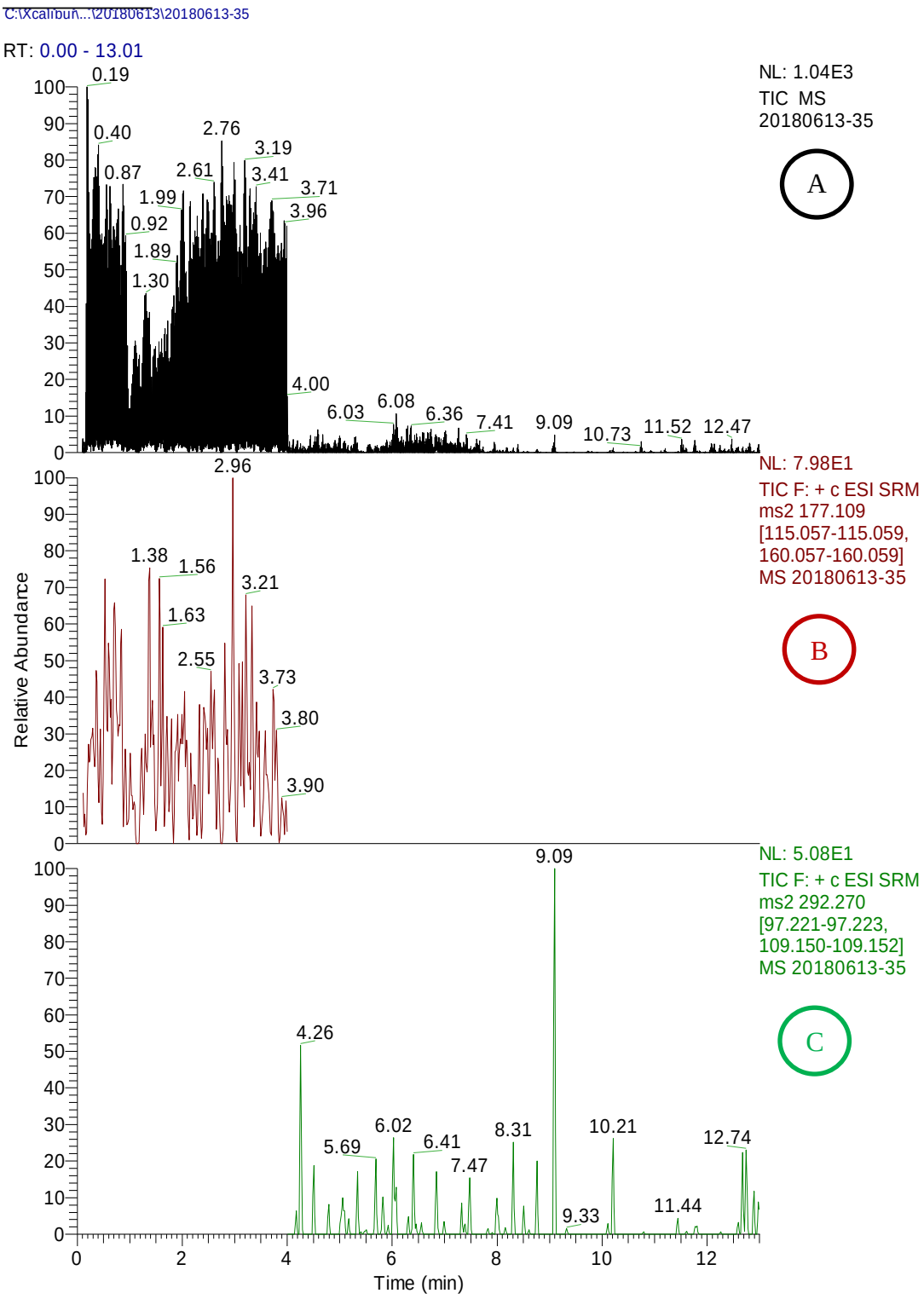

Figure 2.1 Chromatograms of Solvent (Methanol) Blank for (A) Total Ion Chromatogram, (B) Serotonin, and (C) Internal Standard [Testosterone]

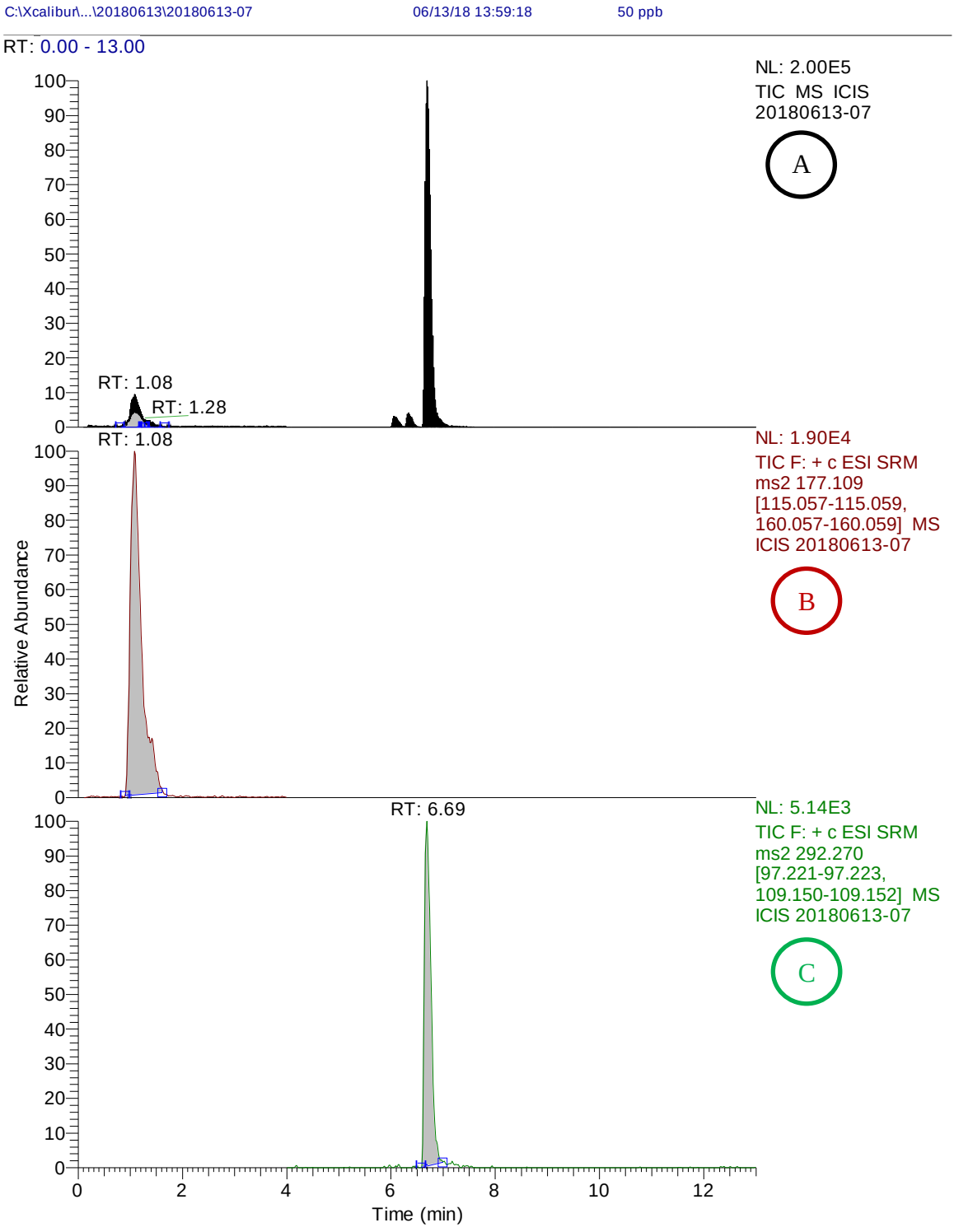

Figure 2.2 Chromatograms of (A) Total Ion Chromatogram (TIC), (B) Serotonin Standard in Methanol, and (C) Internal Standard [Testosterone] in Methanol

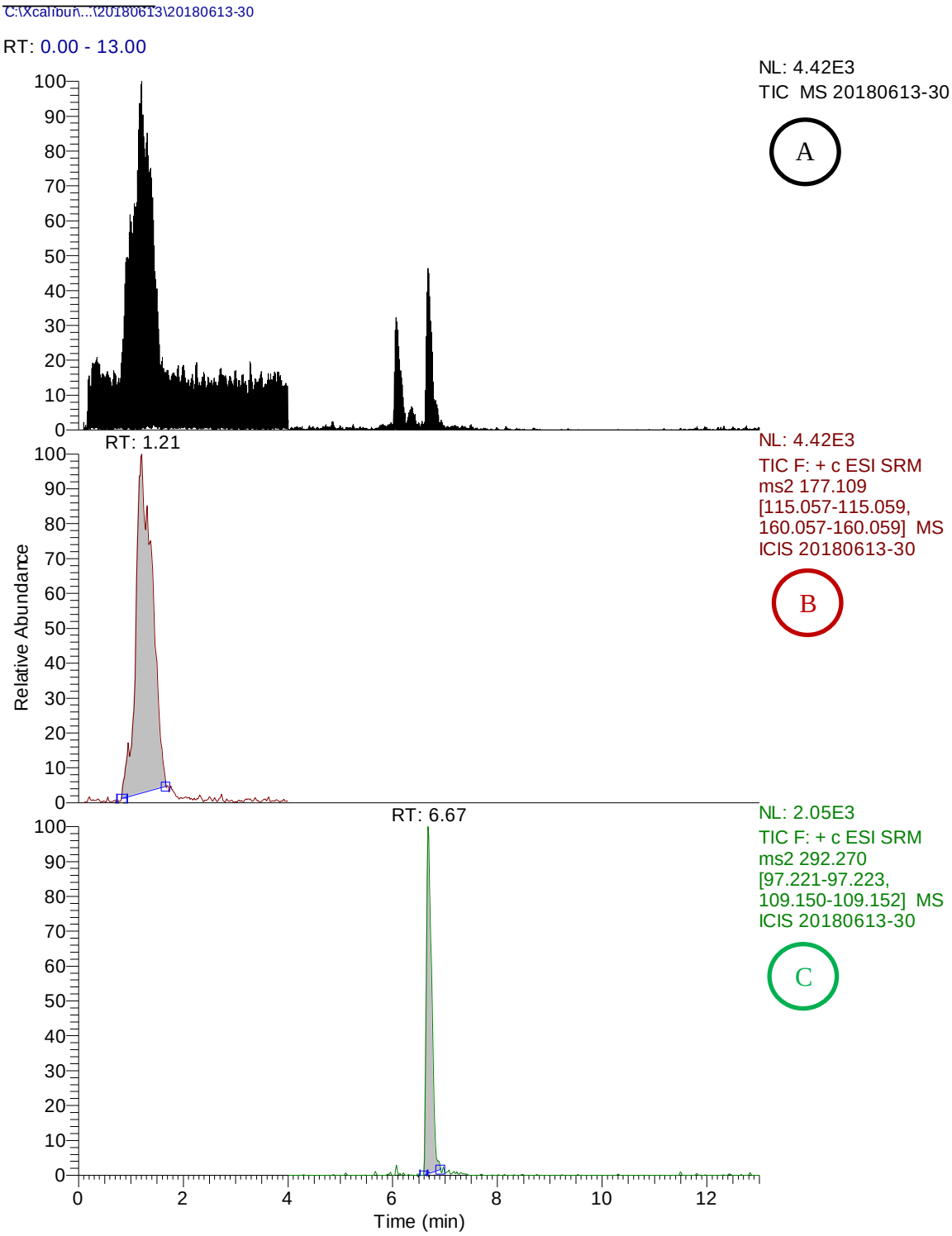

Figure 2.3 Chromatograms of (A) Total Ion Chromatogram (TIC), (B) Serotonin in Serum Sample, and (C) Internal Standard [Testosterone] in Serum Sample

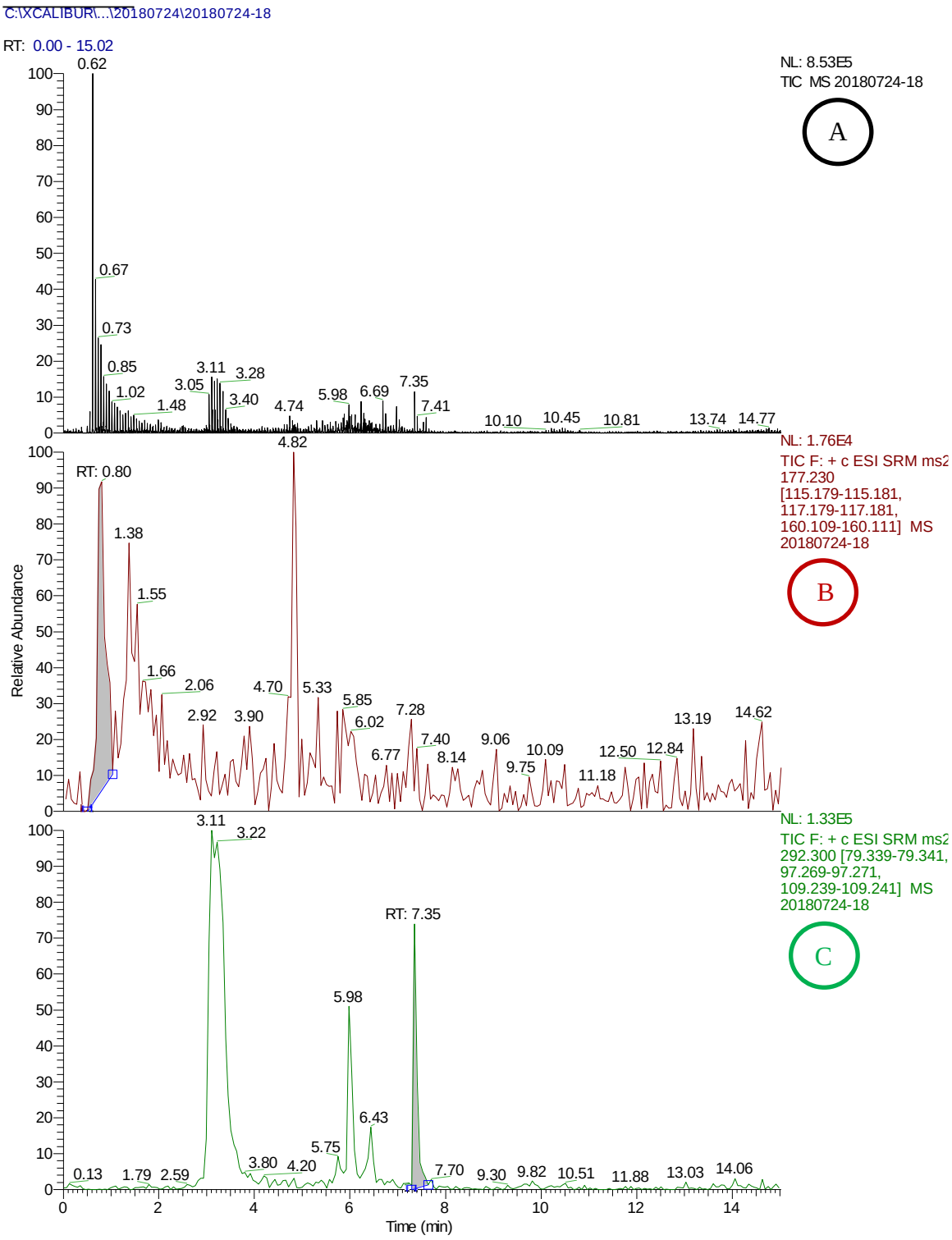

Figure 2.4 Chromatograms of (A) Total Ion Chromatogram (TIC), (B) Serotonin in Urine Sample, and (C) Internal Standard [Testosterone] in Urine Sample

Linearity/LOD/LOQ/Precision/Range/Carryover

The linearity of the calibration curve for serotonin in serum and urine was observed with the correlation coefficient (r^2) range from 0.9973 to 0.9989. The results of the precision obtained for serum using the HESI probe with C_{18} column was 5.66% relative standard deviation (RSD) at 10 ng/mL and 2.84% at 100 ng/mL as displayed in Table 2.3. For urine, it was 5.18% relative standard deviation (RSD) at 10 ng/mL and 2.05% at 100 ng/mL as displayed in Table 2.4.

The limit of detection for serum was determined to be 1.88 ng/mL and the limit of quantification was determined to be 6.27 ng/mL as outlined in Table 2.3. The limit of detection for urine was determined to be 1.76 ng/mL and the limit of quantification for the HESI probe was determined to be 5.86 ng/mL as outlined in Table 2.3.

The FDA guideline on method validation states that when spiked at 10 ppb, the LOD should be greater than or equal to 2 ppb while the LOQ should be greater than or equal to 4 ppb [89]. Our method validation samples have a LOD that is slightly below 2 ppb and LOQ that is within the acceptance criteria of greater than 4 ppb.

Table 2.3 Limit of Detection (LOD), Limit of Quantification (LOQ), and Percent Matrix Effect (ME) for Serotonin in Serum and Urine Using HESI Probe and C_{18} Column

Serotonin (ng/mL)			
Serum	**LOD**	**LOQ**	**ME [%]**
10	1.88	6.27	132.51
Urine			
10	1.76	5.86	10.12

Recovery/Repeatability/Matrix Effects

The mean serotonin recovery values in serum using the HESI probe with C_{18} column ranged from 110.88% to 115.22% as displayed in Table 2.4. In urine, the mean serotonin recovery ranged from 112.10% to 113.15% as displayed in Table 2.4.

In this study, the repeatability analysis for serum was found to be less than 6% and the repeatability analysis for the urine was found to be less than 5.50%. The repeatability results for serum and urine using HESI probe with C_{18} column are summarized in Table 2.4.

The values of ME indicate either ion enhancement (>100%) or ion suppression (<100%) [90]. Serotonin matrix effects were 132.51% for serum using a HESI probe and C_{18} column thus enhancing the sensitivity of the HESI probe (i.e. ion enhancement <100%). Serotonin matrix effects were 10.12% for urine thus reducing the sensitivity of the HESI probe (i.e. ion suppression >100%). In this study, we used internal quality control (QC) procedures to ensure the quality of the data for each set of samples. This QC procedure analyzed and measured the standards using quality control samples which were then followed by the analysis of reagent blanks and sample blanks.

A study that analyzed serotonin in urine via LC-MS/MS obtained similar results with percent recovery from 98 to 104% and percent RSD from 2.5 to 4.3% [91]. Another study conducted by Maeda et al., analyzed serotonin in serum via LC-MS and the percent recovery was 100.1% and the percent RSD was 0.8 when spiking the sample with 100 μL/L [92]. Maeda et al., also noted that 70 to 120% recovery and RSD less than 25% were considered accurate data as indicated previously by the FDA as a guideline and our results meet that criteria [92].

The FDA guideline on method validation states that when spiked at 10 ppb, the percent recovery should be between 60 and 115% while the RSD should be less than 22% [89]. Our method validation samples have a percent recovery and RSD that are within the acceptance criteria. The guideline also states that when spiked at 100 ppb, the percent recovery should be between 80 and 110% while the RSD should be less than 11% [89]. Our method validation samples have a percent recovery slightly higher than 110% and our RSD is within the 11% acceptance criteria.

Table 2.4 Mean Recovery and Relative Standard Deviation Values for Serotonin in Serum and Urine Using HESI Probe and C_{18} Column

Serotonin (ng/mL)		
Serum	**Recovery [%]**	**RSD [%]**
10	110.88	5.66
100	115.22	2.84
Urine		
10	113.15	5.18
100	112.10	2.05

White-tailed Deer Serum and Urine Samples

Serotonin serum concentration ranged from 3.20 ng/mL to 60.53 ng/mL and from 2.01 ng/mL to 20.29 ng/mL in urine. A comparison of the C_{18} and HILIC column for serum and urine are displayed in Figures 2.5 and 2.6, respectively. As discussed earlier, the HILIC column was determined to produce false positive results under our conditions. During the method validation, the HILIC column was determined to be unreliable. In serum serotonin levels, the HILIC column detected concentrations nearly three times higher than the C_{18} column. In urine, the HILIC column detected concentrations approximately seven times higher than the C_{18} column. We also compared serum serotonin concentrations and determined that the male serum serotonin concentration is significantly higher than the female serum serotonin concentration ($p\text{-value}<0.05$) as displayed in Figure 2.7. A comparison of female tissues is displayed in Figure 2.8 and it can be noted that female serum serotonin is significantly higher than serotonin found in urine.

It is established that the breeding season begins in September with peak rut being in December followed by late breeding season ending approximately in March [93]. Over this time frame, blood samples were collected during different months of the year, but primarily during the white-tailed deer breeding season. While it is known that hormones in white-tailed deer such as testosterone and estradiol fluctuate during

the breeding season [93], to our knowledge it has not been determined if neurotransmitters also fluctuate during the breeding season. In Figures 2.9 and 2.10 serotonin concentrations by month in female and male white-tailed deer serum is displayed, respectively. The serotonin concentration by month for female white-tailed deer had samples collected for the months of February, June, July, and October. It appears that the breeding season months, February and October, are numerically higher than the summer months, June and July. The serotonin concentration in February is significantly higher than June serum serotonin concentration (p-value<0.05). The serotonin concentrations by month for the male white-tailed deer had samples collected for the months of February, October, and December. These three months of the breeding season serotonin concentrations appear to remain fairly constant across these months. There was no significant difference across the months sampled for females and males based on the current data.

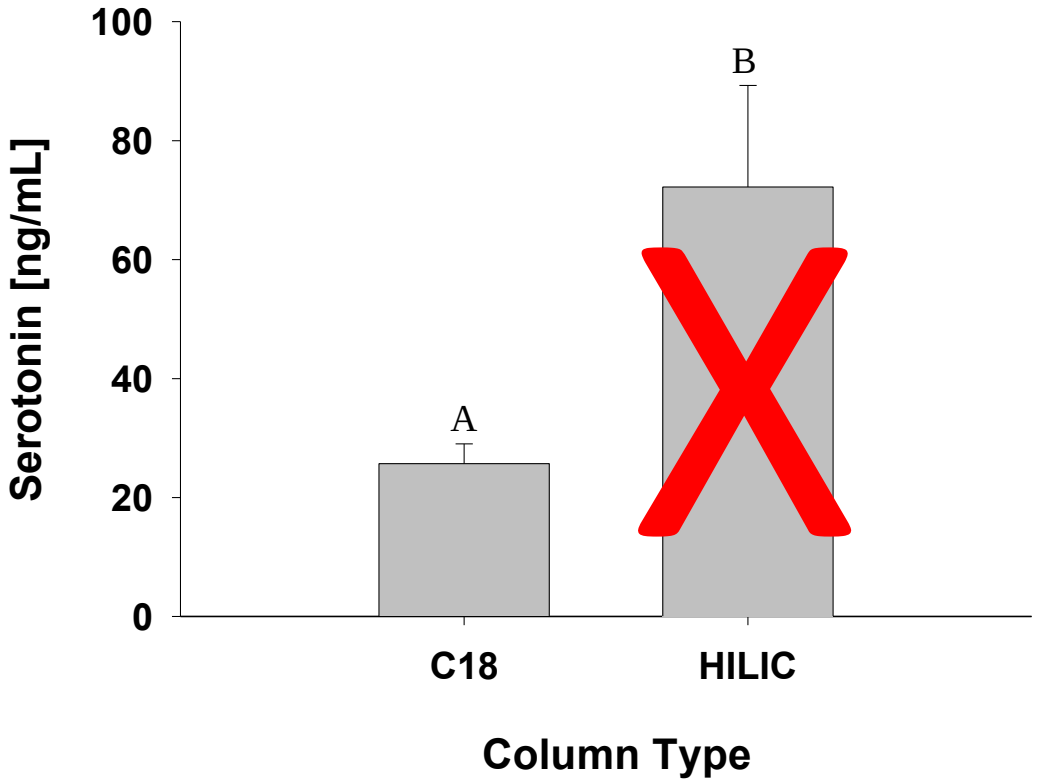

Figure 2.5 A Comparison of the Level of Serotonin Expressed as ng/mL in Serum Samples from White-tailed Deer Using a HESI Probe with both the C_{18} and HILIC Columns

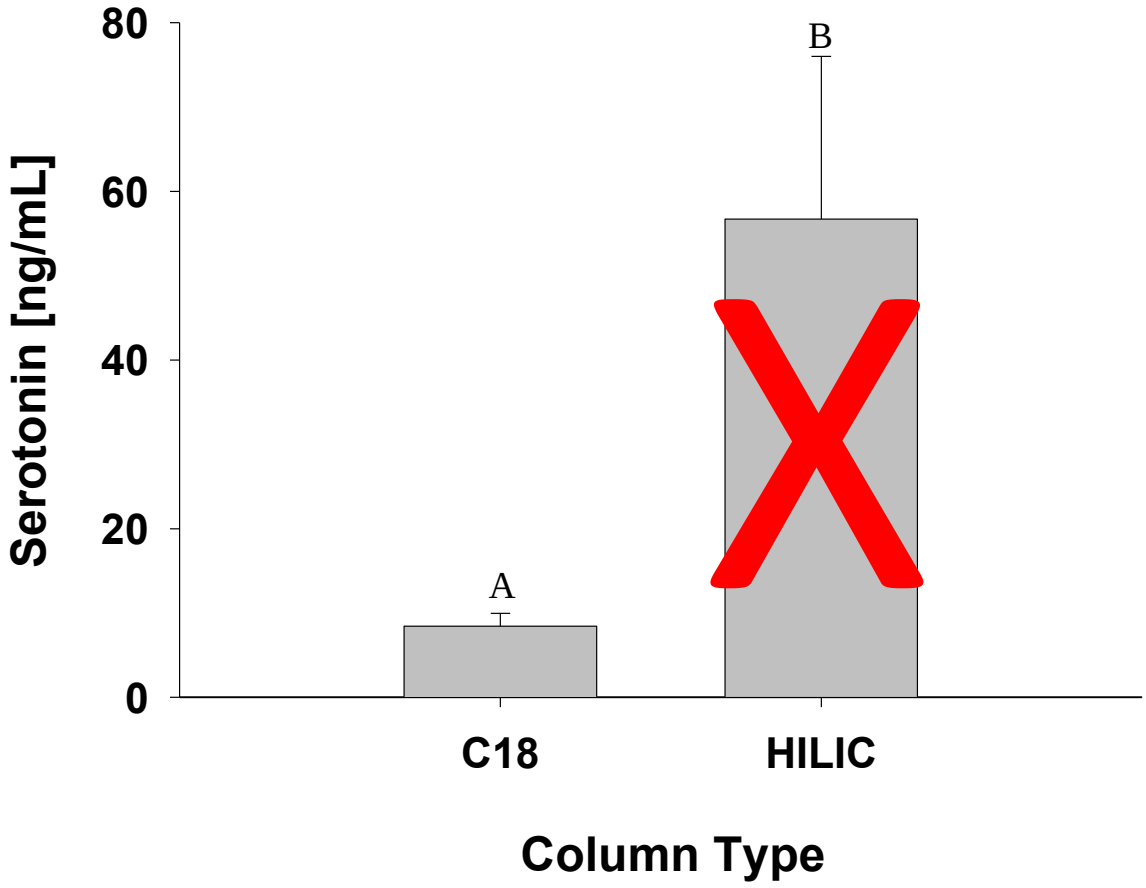

Figure 2.6 A Comparison of the Level of Serotonin Expressed as ng/mL in Urine Samples from White-tailed Deer Using a HESI Probe with both the C_{18} and HILIC Columns

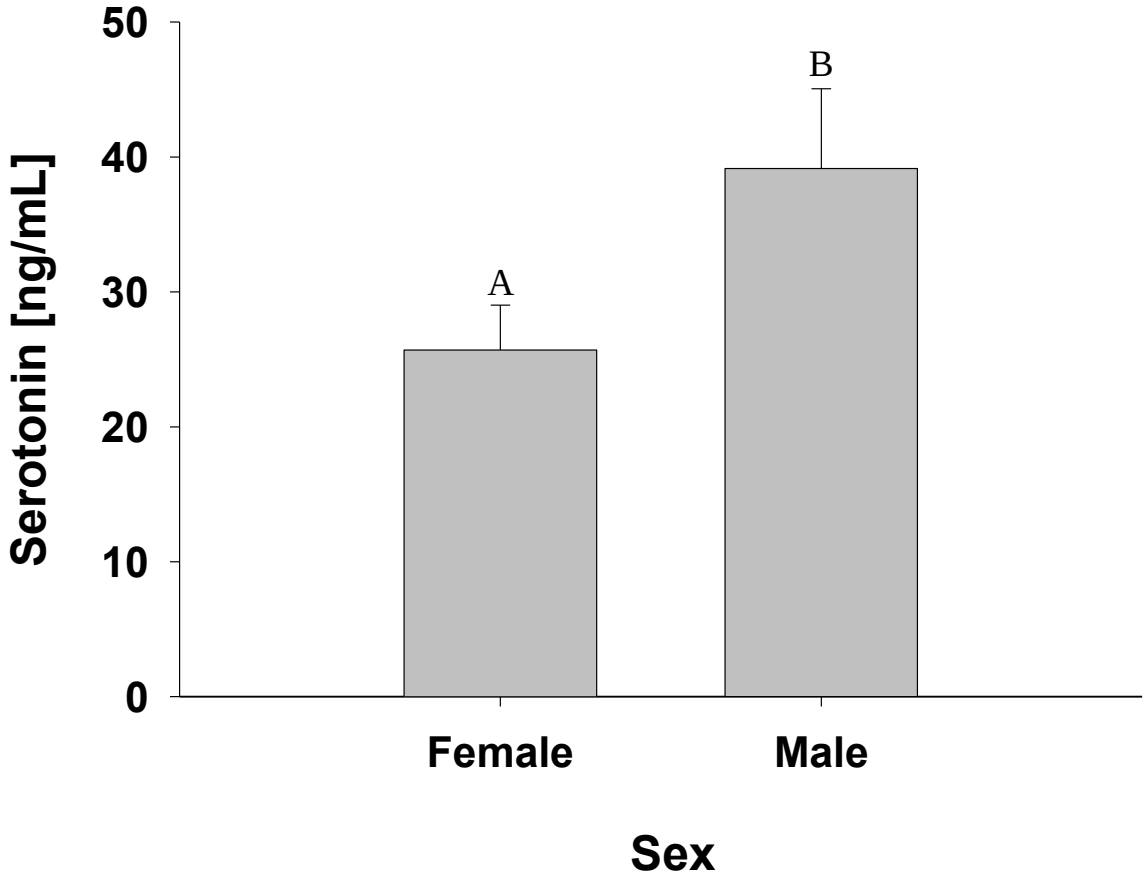

Figure 2.7 A Comparison of White-tailed Deer Serum Serotonin Concentrations by Sex Using a HESI Probe with the C_{18} Column

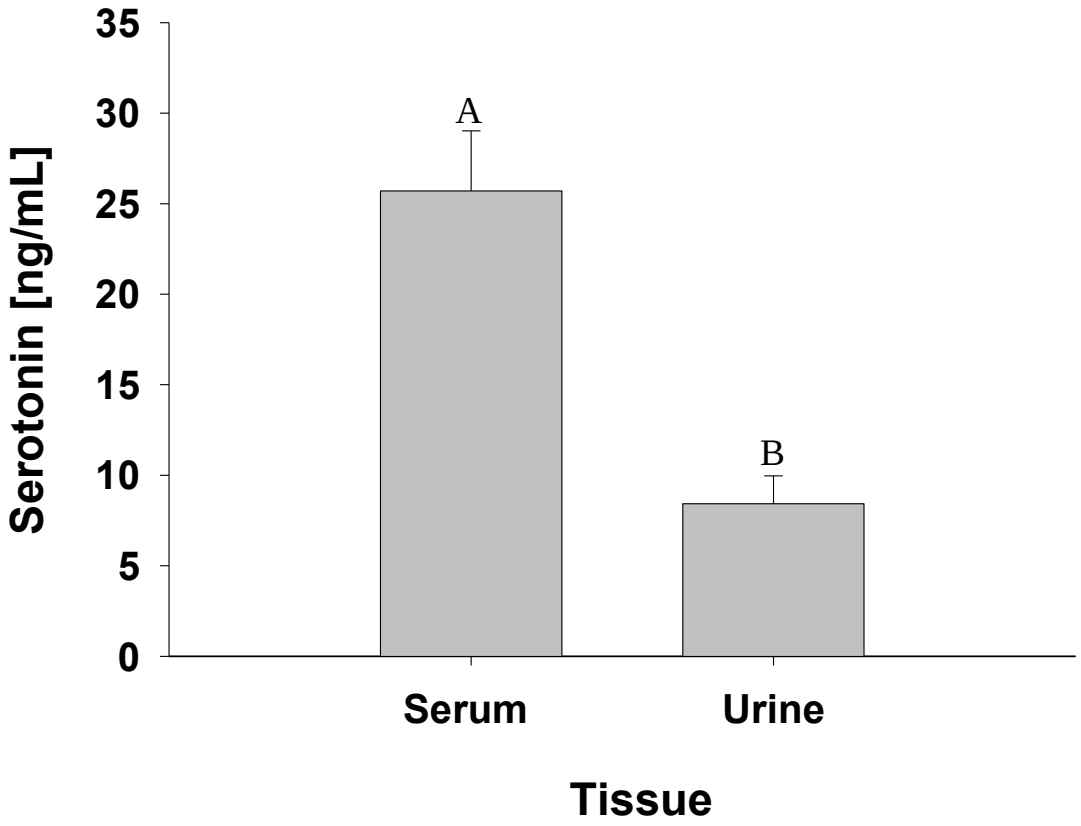

Figure 2.8 A Comparison of the Level of Serotonin Expressed as ng/mL in Serum and Urine Samples from Female White-tailed Deer Using a HESI Probe with the C_{18} Column

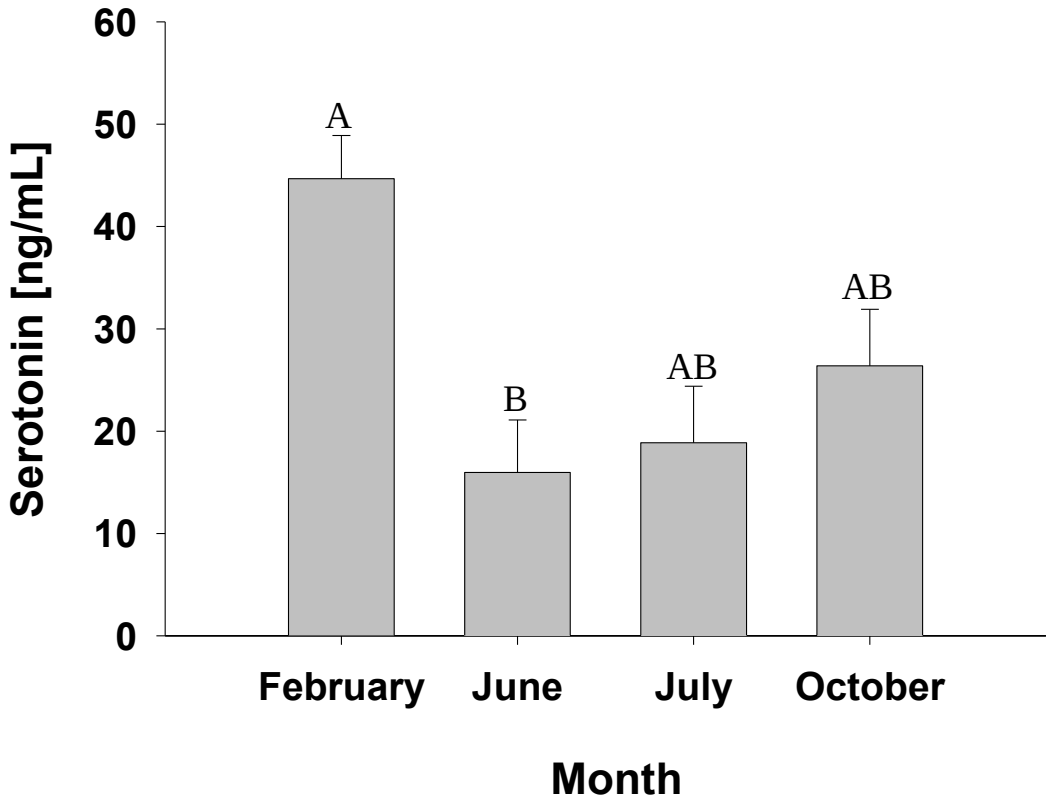

Figure 2.9 Serotonin Concentrations by Month in Female White-tailed Deer Serum Using a HESI Probe with the C_{18} Column

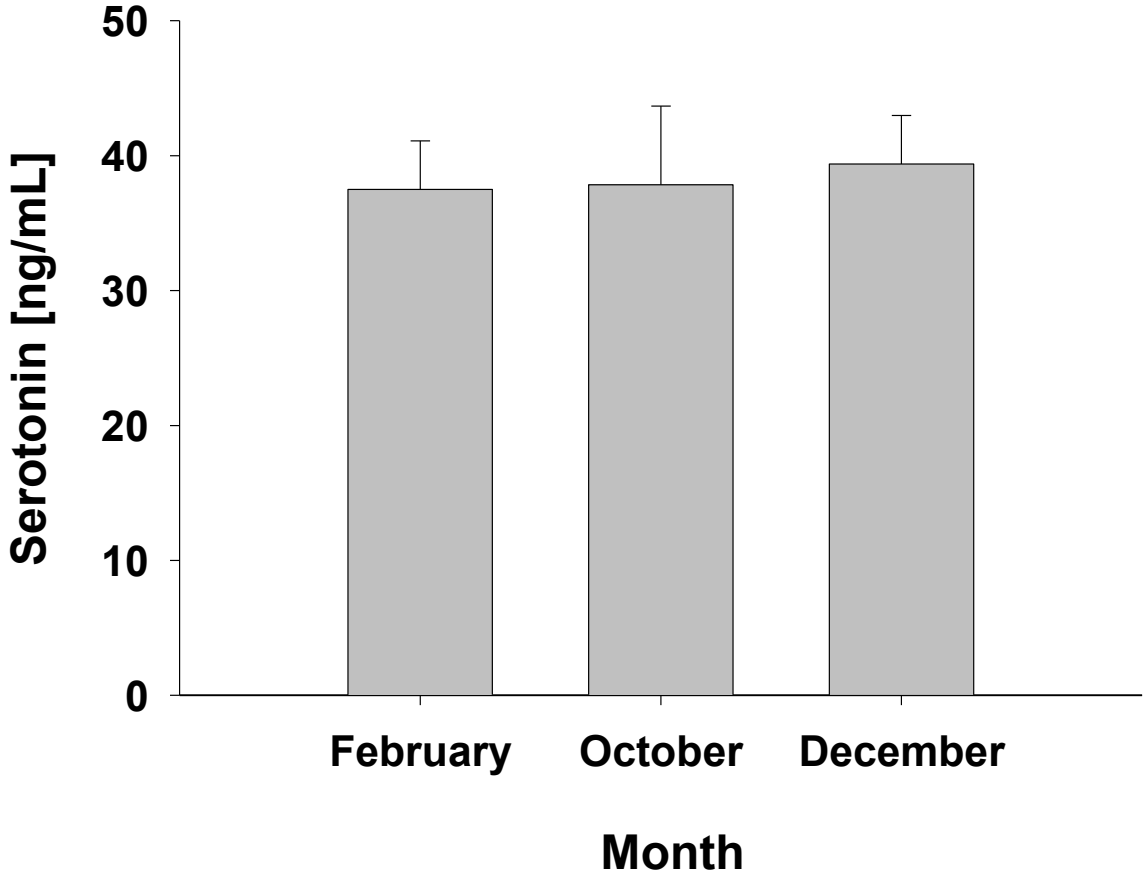

Figure 2.10 Serotonin Concentrations by Month in Male White-tailed Deer Serum Using HESI Probe with the C_{18} Column

Conclusion

In summary, a quantitative liquid chromatography-mass spectrometry method was optimized for the detection and confirmation of 5-hydroxytryptamine (5-HT) in white-tailed tissues. We developed a method for the determination of serotonin in white-tailed deer tissues using a C_{18} column and a HESI probe. This has allowed us to develop baseline, "normal", levels of the monoamine neurotransmitter, serotonin. This "normal" range will allow future studies to characterize serotonin changes in chronic wasting disease cases which will eventually allow for the development of a minimally invasive pre-screening diagnostic indicator for CWD. The baseline serotonin

concentrations can also be used as a biomarker for herd health in white-tailed deer. Further research needs to be done to increase the sample size of this baseline data and to capture a better idea of how serotonin concentrations fluctuate during the year. Collecting samples across a wide range of ages could also help answer the question if serotonin concentrations vary among juveniles, adults, females and males.

CHAPTER 3

SERUM AND URINE CONCENTRATIONS OF SEROTONIN FOLLOWING EXPOSURE TO ANTIBIOTICS IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS

Abstract

A method for the detection and confirmation of serotonin, 5-hydroxytryptamine (5-HT), in white-tailed tissues was developed and validated in our laboratory. Serum and urine samples were extracted with acetonitrile. Liquid chromatography separation was attained on a Phenomenex C_{18} column with a Security Guard ULTRA guard column with gradient elution using a mobile phase of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 250 μL/minute. Analysis was carried out using liquid chromatography heated electrospray ionization tandem mass spectrometry in the selected reaction monitoring interface.

Mean recovery values ranged from 111% to 115% for serum and 112% to 113% in urine. The limits of detection for serum and urine were 1.88 ng/mL and 1.76 ng/mL respectively. The limits of quantification were 6.27 ng/mL for serum and 5.86 ng/mL for urine. Serotonin concentrations for baseline (control group), CTC 60 day treated with 10- and 45-day withdrawal times, and CTC 120 day treated with 10- and 45-day withdrawal times were determined. In separate studies, we also determined serotonin concentrations in white-tailed deer serum following a single dose exposure to florfenicol and tulathromycin with withdrawal times of 10 days.

In summary, we developed a method for the determination of serotonin in white-tailed deer tissues using a C_{18} column and HESI probe following exposure to chlortetracycline, florfenicol, and tulathromycin. This has allowed us to compare serotonin concentrations of control and treated white-tailed deer. Many studies that look at neurotransmitter changes following exposure to antibiotics are in monogastric animals such as mice, rats, and humans. This study is relevant and provides significant data relative to serotonin concentration changes in a ruminant species.

Introduction

Chlortetracycline has been used as an antibiotic growth promoter (AGP) to aid in the productivity of livestock production and improve the health and well-being of animals [1]. AGP is defined as antibiotics administered to animal feed at sub-therapeutic levels to enhance growth and improve feed efficiency [31]. The mechanism of action of orally ingested AGP are primarily focused on the interactions between the antibiotic and the intestinal gut microbiota that could lead to the reduction of the bacterial community [31]. Chlortetracycline (CTC) a natural occurring tetracycline identified from the fungus Streptomyces *aureofaciens* in 1948 and is active against a wide range of gram-positive and gram-negative bacteria [34]. Florfenicol and tulathromycin are commonly used as broad-spectrum antibiotics that act against most gram-positive and gram-negative bacteria in the agricultural industry [94].

A study by Sullivan determined that a streptomycin and chlortetracycline combination given orally to mice showed there was a significant increase of intestinal serotonin in treated mice but no significant difference between treated groups and controls groups after subcutaneous injection of the antibiotics [61]. The authors hypothesized that the sterilization of bacterial microbiota in the gut due to the combination of streptomycin and chlortetracycline may have increased the quantity of tryptophan available for serotonin synthesis by inhibiting the bacterial metabolism of dietary tryptophan [12]. Sullivan administered broad-spectrum antibiotics (ampicillin, neomycin sulfate metronidazole, and vancomycin) to mice in drinking water for 4 weeks and noticed a decreased expression of TPH1 expression in the treated mice [60]. A study by Yano et al., compared peripheral serotonin in mice with normal populations of gut microbes with germ-free mice and found that enterochromaffin cells from germ-free mice produced less serum serotonin than those mice with normal populations of gut microbes [62]. The Yano et al., study determined that the gut microbiota regulates high levels of peripheral serotonin [62].

The aim of our study is to determine whether the use of AGPs such as chlortetracycline alters serotonin concentrations in white-tailed deer tissue samples.

The use of chlortetracycline as an AGP could indirectly affect serotonin levels which could, in turn, affect a variety of moods that serotonin regulates.

Materials and Methods

All reagents were LC-MS grade unless stated otherwise. Methanol, water, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium formate, acetic acid (≥ 99.7) and formic acid (98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Whatman mini-uniprep syringeless filter devices, microcentrifuge tubes (1.5 mL), and disposable borosilicate glass tubes with a plain end (16x100mm) were also purchased from Fisher Scientific (Pittsburgh, PA, USA).

Standard Preparation

Working standard solutions of steroid internal standard (I.S.) were prepared from 100 µg/mL of D_3-Testosterone as (Cerilliant, Round Rock, TX, USA) stock standard. We prepared 10 µg/mL as intermediate standard and 1 µg/mL as working solution concentration. The diluent solvent was methanol in all cases.

Animal Care and Treatment: Chlortetracycline

This experiment was performed in compliance with the appropriate laws and institutional guidelines approved by the Texas Tech Institutional Animal Care and Use Committee, protocol number 14039-08.

Twenty-four healthy white-tailed does with ages ranging from 1 to 6 years old and weights ranging between 32.70 kg and 54.80 kg were randomly divided into 3 groups: 1 control group and 2 treated groups. All groups were kept in high fenced pens. They were given *ad libitum* access to feed and water throughout the experiment. The animals were randomly assigned to their treatment groups.

The control groups consisted of eleven animals that received feed without CTC. The first treatment group consisted of six animals that received sixty days of chlortetracycline (CTC) treated feed containing 250 mg/kg of CTC. After sixty days, the treated group received control feed without CTC for the duration of the withdrawal period which were ten and forty-five days. The second treatment group consisted of seven animals that received one hundred-twenty days of CTC treated feed containing

250 mg/kg of CTC. After one hundred-twenty days, the treated group received control feed without CTC for the duration of the withdrawal period which were ten and forty-five days. Blood and urine samples were collected for neurotransmitter analysis from each animal on the day of euthanasia during a previous study [35]. Lab personnel, as well as the university's veterinarians, monitored the health of the animals. The samples were stored at –80°C until analysis.

Animal Care and Treatment: Florfenicol

This experiment was performed in compliance with the appropriate laws and institutional guidelines approved by the Texas Tech Institutional Animal Care and Use Committee, protocol number 14039-08.

Fourteen healthy white-tailed does with ages ranging from 1 to 6 years old and weights ranging between 32.70 kg and 46.70 kg were randomly divided into 2 groups: 1 control group and 1 treated group. Both groups were kept in high fenced pens. They were given *ad libitum* access to feed and water throughout the experiment. The animals were randomly assigned to their treatment groups. The treated group consisted of three animals that were administered a single dose intramuscularly of florfenicol at 20 mg/kg (3 mL/100 lb.) of body weight. The control group consisted of eleven does. Lab personnel as well as the university's veterinarians monitored the health of the animals. Blood samples were collected ten days after exposure for neurotransmitter analysis from each animal on the day of euthanasia. The samples were stored at –80°C until analysis.

Animal Care and Treatment: Tulathromycin

This experiment was performed in compliance with the appropriate laws and institutional guidelines approved by the Texas Tech Institutional Animal Care and Use Committee, protocol number 14039-08.

Eighteen healthy white-tailed does with ages ranging from 1 to 6 years old and weights ranging between 32.70 kg and 49.50 kg were randomly divided into 2 groups: 1 control group and 1 treated group. Both groups were kept in high fenced pens. They were given *ad libitum* access to feed and water throughout the experiment. The

animals were randomly assigned to their treatment groups. The treated group consisted of four animals that were administered a single dose of tulathromycin intramuscularly at 2.5 mg/kg (1.1 mL/100 lb.) of body weight. The control group consisted of eleven does. Lab personnel, as well as the university's veterinarians, monitored the health of the animals. Blood samples were collected ten days after exposure for neurotransmitter analysis from each animal on the day of euthanasia. The samples were stored at –80°C until analysis.

Serum Sample Preparation

Serum preparation involved centrifuging the serum separator tube (SST) with the blood sample at 2000 rpm for 15 minutes at 10°C. Serum was removed using transfer pipettes and placed in Eppendorf tubes. The samples were then stored at -80°C until analysis.

Serotonin determination in serum was performed using a modified version of Dong et al., techniques [81]. An aliquot of 50 μL serum sample was put into a microcentrifuge tube. 5μL steroid I.S. (1μg/mL in methanol) and 195 μL ice-cold acetonitrile were added to precipitate protein and other coextractives. It was vortexed for 30 seconds and then centrifuged at 10,000 rpm for 10 minutes at 4°C. 150 μL of the supernatant was transferred to a syringeless filter device and diluted with 150 μL water.

Urine Sample Preparation

Urine pre-sample preparation involved taking 1 mL urine and centrifuging at 10,000 rpm for 10 minutes at 4°C. Steroid and neurotransmitter determination in urine was performed using a modified version of Zhai et al., techniques [82]. 5 μL of 1μg/mL I.S. steroids were added to an aliquot of 200 μL urine supernatant in a microtube. It was vortexed for 30 seconds and 195 μL ice-cold acetonitrile was added to precipitate protein and other extractives. This mixture was vortexed for 2 minutes and centrifuged at 5200 rpm for 10 minutes at 4°C. 200 μL of the supernatant was transferred to a disposable borosilicate glass tube (50 mL) and evaporated to dryness

on a water bath at 35°C under a protective atmosphere of nitrogen. The residue was reconstituted with 100 μL 0.1% aqueous acetic acid-methanol mixture (90:10 v/v).

Chromatographic Conditions

Conditions for white-tailed deer serum and urine were optimized using a heated electrospray ionization (HESI) probe for the detection of serotonin. Chromatographic separation was carried out using a refrigerated auto-sampler (CTC PAL) system with a Phenomenex Nx 3 μ particle size; C_{18} column (150mm length x ~2mm) with a Security Guard ULTRA guard column (Phenomenex, USA) on column heater coupled to a Quantum Access MAX triple stage quadrupole (TSQ) mass spectrometer (Thermo Scientific) for optimization and validation.

The column temperature was set to 30°C. Solvents that constituted the mobile phase for the C_{18} column were water with 0.1% formic acid (A), acetonitrile with 0.1% formic acid (B). Mobile phase composition (A:B; v/v) was 90:10 at 0 min, 90:10 at 3 min, 10:90 at 6 min, 10:90 at 9 min, 90:10 at 12 min, and 90:10 at 15 min with a flow rate of 0.25 mL/min and an injection volume of 20 μL.

LC-MS/MS Analysis

MS/MS conditions for analysis of serotonin and the internal standard [D_3-testosterone] were optimized using HESI probe in positive polarity. A 5μg/mL standard was used to determine the optimum counts per seconds (cps) in MS and MS/MS scans. The MS parameters used for the HESI probe and C_{18} column are outlined in Table 3.1. Compound-specific mass spectrometer parent and product ion settings are presented in Table 3.2. White-tailed deer sample validation analyses were carried out for the HESI probe. The comparative results for these techniques were documented and discussed below.

Table 3.1 Mass Spectrometer Parameters for HESI Probe Using C_{18} Column

MS parameters	**HESI**
Column Type	C_{18}
Polarity	Positive
Vaporizer Temperature (°C)	317
Capillary Temperature (°C)	333
Sheath Gas Pressure [Arb], Nitrogen	40
Auxiliary Gas Pressure [Arb], Nitrogen	12
Peak Width Q1, Q3 (FWHM)	0.70
Collision Gas Pressure (mTorr), Argon	1.5
Spray Voltage	3500
Scan Method	SRM

Table 3.2 Mass Spectrometer Parameters for HESI Probe Using C_{18} Column

Analyte	Parent Ion (Q1)	Product Ion (Q3)	Collision Energy (v)	Tube Lens (v)	Ions
Serotonin	177.109	115.058	27.848	68.225	Quantifier
		160.058	10.253	68.225	Qualifier
D_3-Testosterone	292.27	109.151	21.629	119.191	Quantifier
		97.222	19.607	119.191	Qualifier

Method Validation

The LC-MS/MS method was validated for control white-tailed deer serum and urine samples. The parameters determined were specificity, selectivity, linearity, system precision, repeatability, matrix effect, and internal quality control.

Method-Specificity/Selectivity

To determine the selectivity and specificity of the method and to demonstrate the absence of interference with the elution of the analyte, we analyzed a solvent blank followed by blank white-tailed deer serum and urine extracts. This was followed by analysis of a reference solvent standard and analysis of white-tailed deer serum and urine matrix standards. The blank white-tailed deer matrix sample was spiked with a low level, 10 ng/mL, of serotonin prior to processing and analysis.

Method-Linearity/Limit of Detection/Limit of Quantification/Precision/Range/Carryover

White-tailed deer serum and urine were used for matrix-fortified calibration curves. To our knowledge, there are no established neurotransmitter concentration levels in white-tailed deer serum and urine. The selected concentration ranges for the solvent and matrix-matched calibration standards for serotonin were: 1, 5, 10, 25, 50, 100, and 250 ng/mL (ppb). The limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated based on the blank determination using the

formula LOD = $X_{b1} + 3S_{b1}$ and LOQ = $X_{b1} + 10S_{b1}$ where X_{b1} is the mean concentration of the blank and S_{b1} is the standard deviation of the blank [83]. The LOD was set based on the measurement of seven replications of blank white-tailed deer serum and urine and spiked with 10 ng/mL. The standard deviation from the spiked samples were multiplied by 3 (3 x SD) to establish the LOD used for this study. The standard deviation of the spiked samples was multiplied by ten (10 x SD) to establish the LOQ for this study.

Method-Recovery/Repeatability/Matrix Effects

Two concentrations of serotonin were used to establish recovery and repeatability of the current method. Spiked white-tailed deer serum and urine samples were used to determine method recovery. For this determination, untreated white-tailed deer serum and urine samples were fortified with 10 and 100 ng/mL of serotonin. This concentration was replicated seven times. The samples were analyzed for the concentration of serotonin. Blank white-tailed deer serum and urine sample extracts were used as a diluent to estimate matrix effects. To determine the matrix effects, the mean area counts of the serotonin in the spiked samples were divided by the mean area counts of serotonin standard solutions and multiplied by 100.

Method-Internal Quality Control

The internal quality control of the system was performed by injecting 25 ng/mL standard as an initial calibration verification (ICV). After every tenth sample injection in the sequence a continuum calibration verification (CCV) serotonin standard of 25 ng/mL was injected and analyzed.

Statistical Analysis

Statistical differences between means were evaluated with analysis of variance (ANOVA) test and with two-tailed Student's *t*-test using JMP statistical software. *P* values less than 0.05 were taken to be statistically significant. If there was a significant difference after the ANOVA test, a Tukey HSD test was conducted to tell where the significant difference was.

Results and Discussion

In an effort to analyze serotonin levels in tissues from white-tailed deer that have been treated with antibiotics, we utilized liquid-liquid extraction. As well as liquid chromatographic separation followed by MS product and parent ion pattern for qualitative and quantitative determination of serotonin concentrations in blood and urine. To our knowledge, there is no data available characterizing serotonin levels in white-tailed deer treated with antibiotics.

Based on our previous protocol and our derived white-tailed deer baseline serotonin data, we used the HESI probe with the C_{18} column for the quantitative and qualitative determination of serotonin in white-tailed deer serum and urine samples.

Method Validation

In this study, the mobile phase of 0.1% formic acid in water and acetonitrile provided clear chromatographic separation of serotonin in serum and urine samples. The combination of the mobile phase and the chromatographic conditions described in Chapter 2 for serotonin facilitated the retention time of 1.08 minutes in serum and 1.21 minutes in urine. Selectivity was verified by analyzing seven replicates of blank white-tailed deer serum and urine samples and it was determined that there were no internal interfering substances from the samples at the same m/z ratio of serotonin retention times Figures 3.1-4.

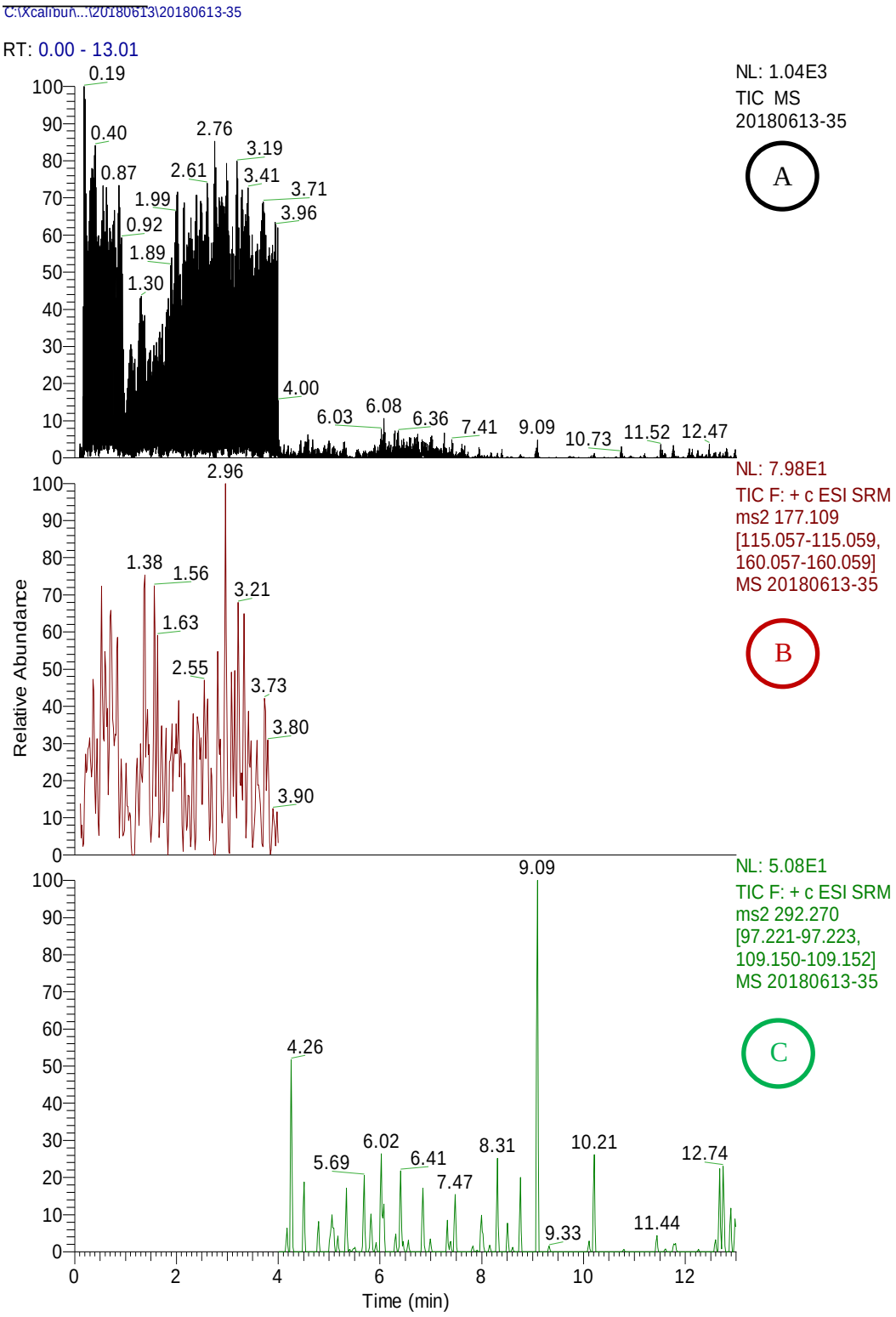

Figure 3.1 Chromatograms of Solvent (Methanol) Blank for (A) Total Ion Chromatogram, (B) Serotonin, and (C) Internal Standard [Testosterone]

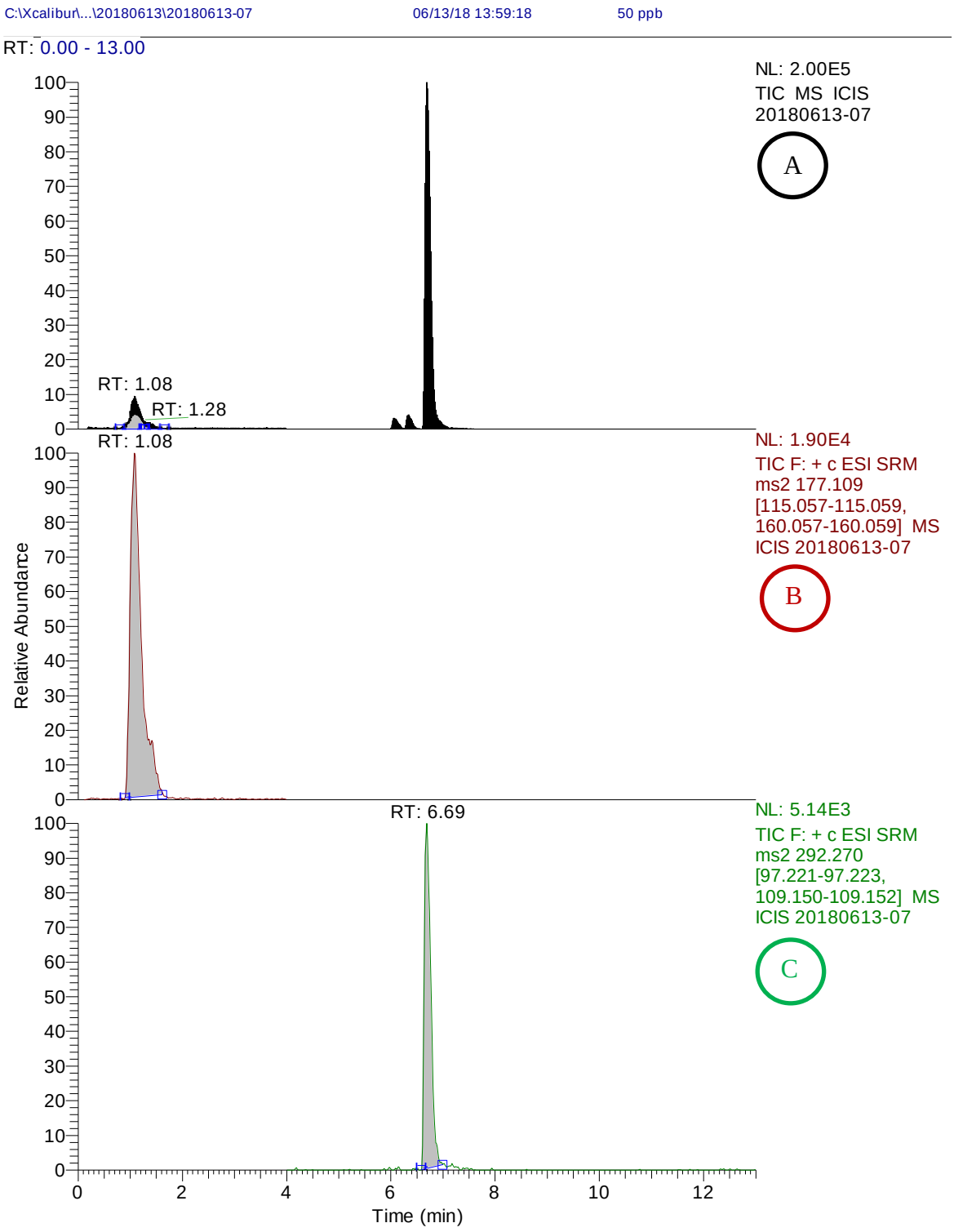

Figure 3.2 Chromatograms of (A) Total Ion Chromatogram (TIC), (B) Serotonin Standard in Methanol, and (C) Internal Standard [Testosterone] in Methanol

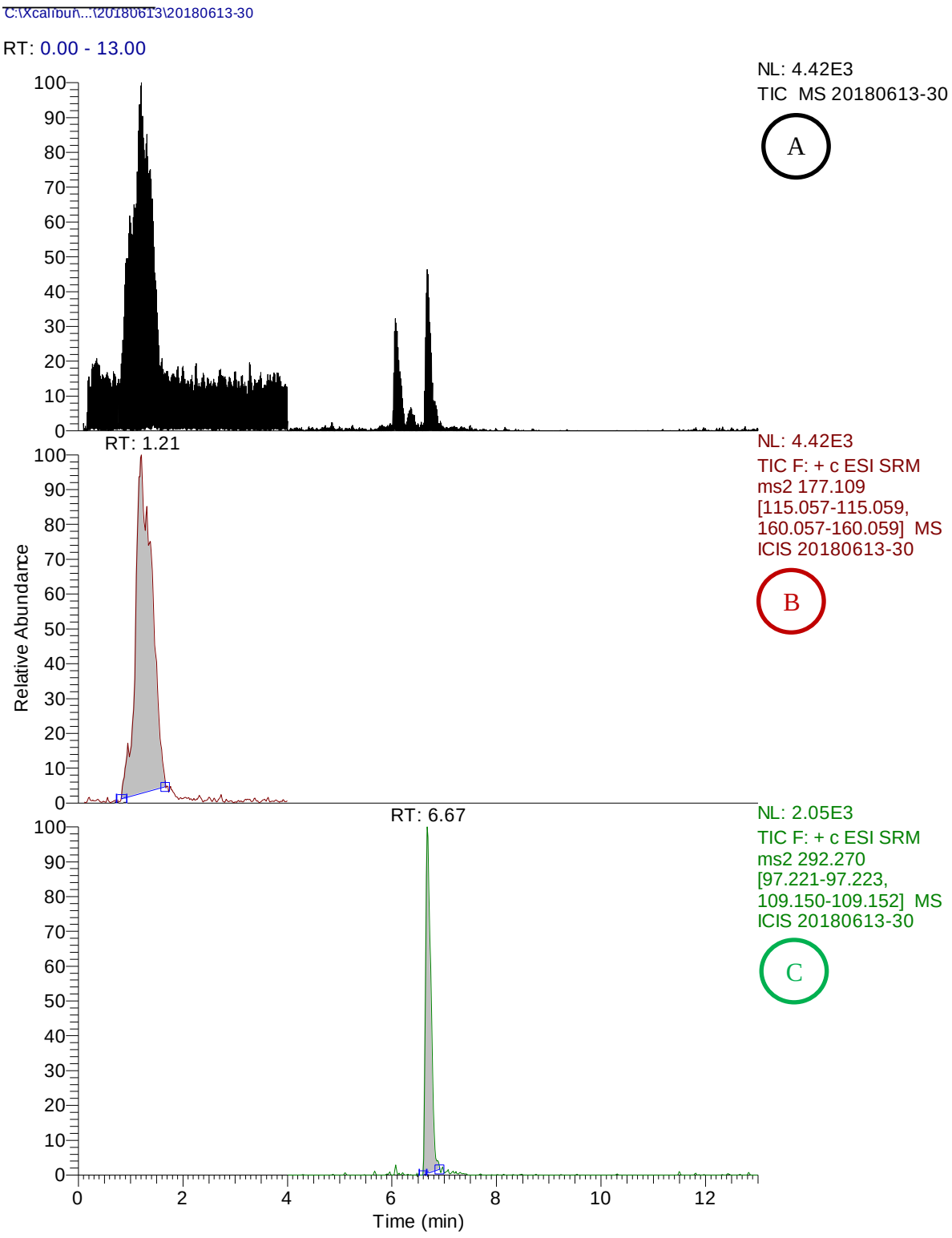

Figure 3.3 Chromatograms of (A) Total Ion Chromatogram (TIC), (B) Serotonin in Serum Sample, and (C) Internal Standard [Testosterone] in Serum Sample

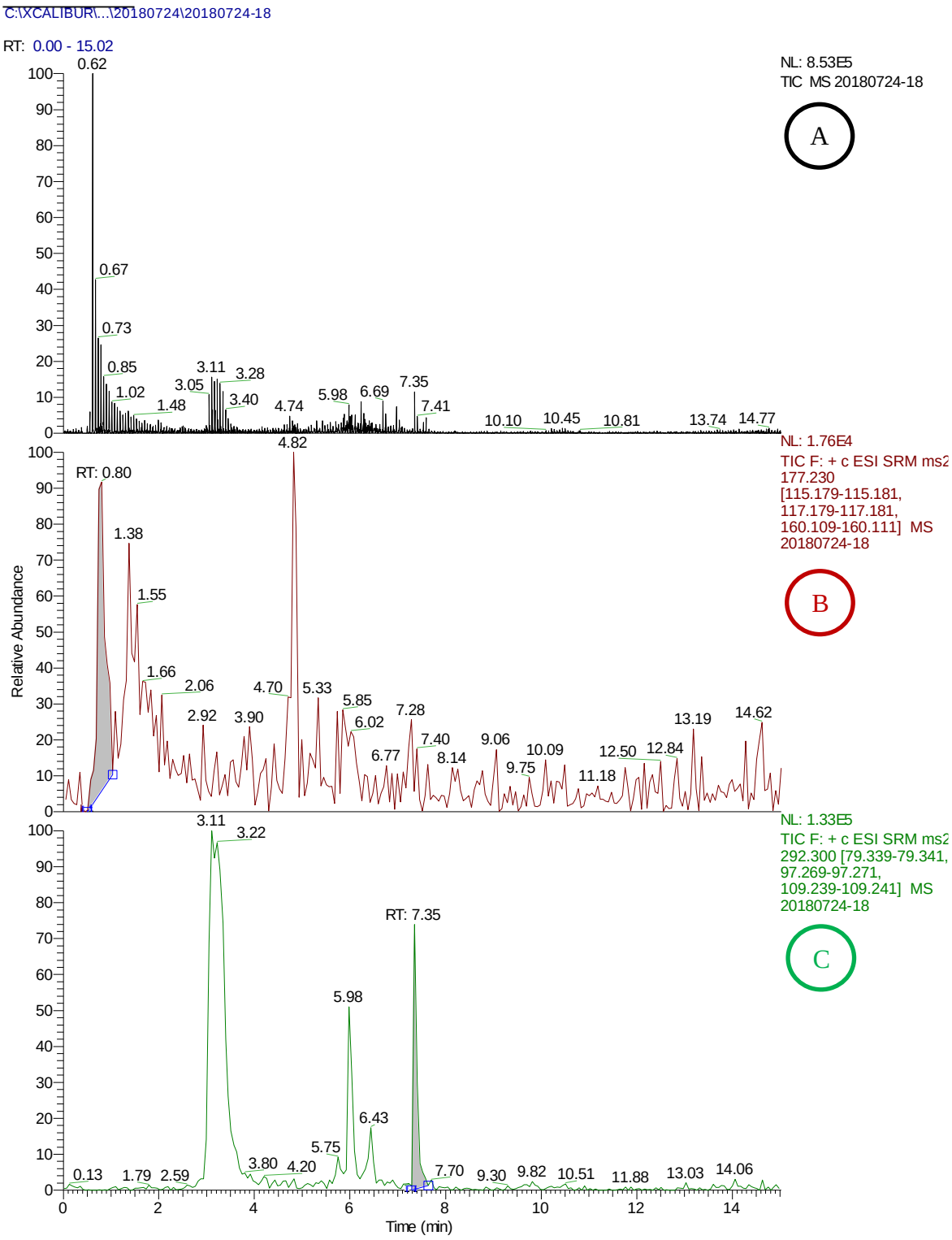

Figure 3.4 Chromatograms of (A) Total Ion Chromatogram (TIC), (B) Serotonin in Urine Sample, and (C) Internal Standard [Testosterone] in Urine Sample

Linearity/LOD/LOQ/Precision/Range/Carryover

The linearity of the calibration curve for serotonin in serum and urine was observed with the correlation coefficient (r^2) range from 0.9973 to 0.9989. The results of the precision obtained for serum using the HESI probe with C_{18} column was 5.66% relative standard deviation (RSD) at 10 ng/mL and 2.84% at 100 ng/mL as displayed in Table 3.4. For urine it was 5.18% relative standard deviation (RSD) at 10 ng/mL and 2.05% at 100 ng/mL as displayed in Table 3.4. The limit of detection for serum was determined to be 1.88 ng/mL and the limit of quantification was determined to be 6.27 ng/mL as outlined in Table 3.3. The limit of detection for urine was determined to be 1.76 ng/mL and the limit of quantification for the HESI probe was determined to be 5.86 ng/mL as outlined in Table 3.3.

The FDA guideline on method validation states that when spiked at 10 ppb, the LOD should be greater than or equal to 2 ppb while the LOQ should be greater than or equal to 4 ppb [89]. Our method validation samples have a LOD that is slightly below 2 ppb and LOQ that is within the acceptance criteria of greater than 4 ppb.

Table 3.3 LOD, LOQ, and Percent Matrix Effect (ME) for Serotonin in Serum and Urine Using HESI Probe and C_{18} Column

Serotonin (ng/mL)			
Serum	**LOD**	**LOQ**	**ME [%]**
10	1.88	6.27	132.51
Urine			
10	1.76	5.86	10.12

Recovery/Repeatability/Matrix Effects

The mean serotonin recovery values in serum using the HESI probe with C_{18} column ranged from 110.88% to 115.22%, while the percent relative standard deviation (RSD) ranged from 2.84% to 5.66% as displayed in Table 3.4. In urine, the mean serotonin recovery ranged from 112.10% to 113.15%, while the percent RSD ranged from 2.05% to 5.18% as expressed in Table 3.4. In this study, the repeatability

analysis for serum was found to be less than 6% and the repeatability analysis for the urine was found to be less than 5.50%. The repeatability results for serum and urine using HESI probe with C_{18} column are summarized in Table 3.3.

The values of ME indicate either ion enhancement (>100%) or ion suppression (<100%) [90]. Serotonin matrix effects were 132.51% for serum using HESI probe and C_{18} column thus enhancing the sensitivity of the HESI probe (i.e. ion enhancement <100%). Serotonin matrix effects were 10.12% for urine thus reducing the sensitivity of the HESI probe (i.e. ion suppression >100%). In this study, we used internal quality control (QC) procedures to ensure the quality of the data for each set of samples. This QC procedure analyzed and measured the standards using quality control samples which were then followed by the analysis of reagent blanks and sample blanks.

The FDA guideline on method validation states that when spiked at 10 ppb, the percent recovery should be between 60 and 115% while the RSD should be 22% [89]. Our method validation samples have a percent recovery and RSD that are within the acceptance criteria. The guideline also states that when spiked at 100 ppb, the percent recovery should be between 80 and 110% while the RSD should be 11% [89]. Our method validation samples have a percent recovery slightly higher than 110% and our RSD is within the 11% acceptance criteria.

Table 3.4 Mean Recovery and Relative Standard Deviation (RSD) Values for Serotonin in Serum and Urine Using HESI Probe and C_{18} Column

Serotonin (ng/mL)		
Serum	**Recovery [%]**	**RSD [%]**
10	110.88	5.66
100	115.22	2.84
Urine		
10	113.15	5.18
100	112.10	2.05

White-tailed Deer Serum and Urine Samples

Baseline (control) serotonin serum concentration ranged from 3.20 ng/mL to 43.15 ng/mL and from 2.01 ng/mL to 20.29 ng/mL in urine as displayed in Figures 3.5 and 3.6, respectively. The CTC 60 day treated group with a withdrawal time of 10 days had serotonin serum concentrations that ranged from 1.85 ng/mL to 36.78 ng/mL and from 7.78 ng/mL to 42.84 ng/mL in the 45-day withdrawal time. In urine, serotonin concentrations for the CTC 60 day treated group with a withdrawal time of 10 days ranged from 4.41 ng/mL to 21.22 ng/mL and from 15.41 ng/mL to 72.36 ng/mL in the 45-day withdrawal time. The CTC 120 day treated group with a withdrawal time of 10 days had serotonin serum concentrations that ranged from 13.91 ng/mL to 38.60 ng/mL and from 34.49 ng/mL to 55.02 ng/mL in the 45-day withdrawal time. In urine, serotonin concentrations for the CTC 60 day treated group with a withdrawal time of 10 days ranged from 4.78 ng/mL to 14.53 ng/mL and from 3.68 ng/mL to 6.92 ng/mL in the 45-day withdrawal time. There was no significant difference in serum serotonin concentrations. But there was a significant difference among the urine serotonin concentrations (p-value<0.05). It was determined that the significant difference was between the CTC 60 day treated group with a withdrawal time of 45 days and all other groups, control and treated.

The microbiome can alter neurological function directly by controlling the concentrations of neurotransmitters in the brain and the periphery. One specific example of this direct mechanism is a decrease in peripheral serum serotonin levels in the absence of gut microbiota. This decrease in serum serotonin levels "corresponds to lower levels of 5-HT metabolites and precursors in the intestinal contents and urine" [63, 66, 67].

Yano et al., compared peripheral serotonin in mice with normal populations of gut microbes with germ-free mice and found that enterochromaffin cells from germ-free mice produced less serotonin serum and colon than those mice with normal populations of gut microbes [62]. This falls in line with what Sampson and Mazmanian stated about there being a decrease in peripheral serum serotonin levels in the absence of gut microbiota.

There have been several studies done that have looked at antibiotic treatment on peripheral serotonin. Sullivan administered antibiotics to mice and rats twice daily, orally by stomach tube. The results for the streptomycin and chlortetracycline combination given orally showed there was a significant increase of intestinal serotonin in treated mice [61]. This study hypothesized that the sterilization of bacterial flora in the gut due to the combination of streptomycin and chlortetracycline may have boosted the amount of tryptophan available for serotonin synthesis by inhibiting the bacterial metabolism of dietary tryptophan [61].

Ge et al., administered broad-spectrum antibiotics (ampicillin, neomycin sulfate metronidazole, and vancomycin) to ten mice in drinking water for 4 weeks. It was determined that there was a depletion in microbiota by the antibiotic combination and this led to a deficit of colonic serotonin in mice [60]. This study also concluded that there was a depletion of microbiota due to antibiotics, but they saw a decrease in colonic serotonin compared to the rise in intestinal serotonin that Sullivan saw in their study.

Our results are different than previous studies [60-62] have observed and that may be for a significant number of reasons. The species difference [mice and rats] is one reason for the difference between our data and the reports of the effects of antibiotics on gut microbiota and serotonin. These represent monogastric species. In contrast, the white-tailed deer represents a ruminant model. So, it is understandable to find differences in the gut microbiota. Urine serotonin concentrations following exposure to chlortetracycline in feed is displayed in Figure 3.6. It was determined that there was a significant difference among treatment groups ($p\text{-value}<0.05$). The CTC 60 day treated group with a withdrawal period of 45 days was significantly different from all other groups, control and treated. All groups were treated the same in terms of animal care, so this spike in serotonin is interesting. There may be environmental factors that influenced the rise in serotonin for this group, but there is no definitive reason why that is the only group with high levels of serotonin. In this study, the sample size in the treated groups is relatively small. We would need a larger sample size to increase our confidence in our concentration values.

In addition to the chlortetracycline treatment groups, two other treatment groups were included in this antibiotic study. In the florfenicol treated group, the serum serotonin concentration ranged from 2.10 ng/mL to 14.28 ng/mL as displayed in Figure 3.7. In the tulathromycin treated group, the serum serotonin concentration ranged from 7.54 ng/mL to 20.52 ng/mL as displayed in Figure 3.7. The control group ranged from 3.20 ng/mL to 43.15 ng/mL as displayed in Figure 3.7. It was determined that there is a statistical difference between the control group and florfenicol (p-value<0.05).

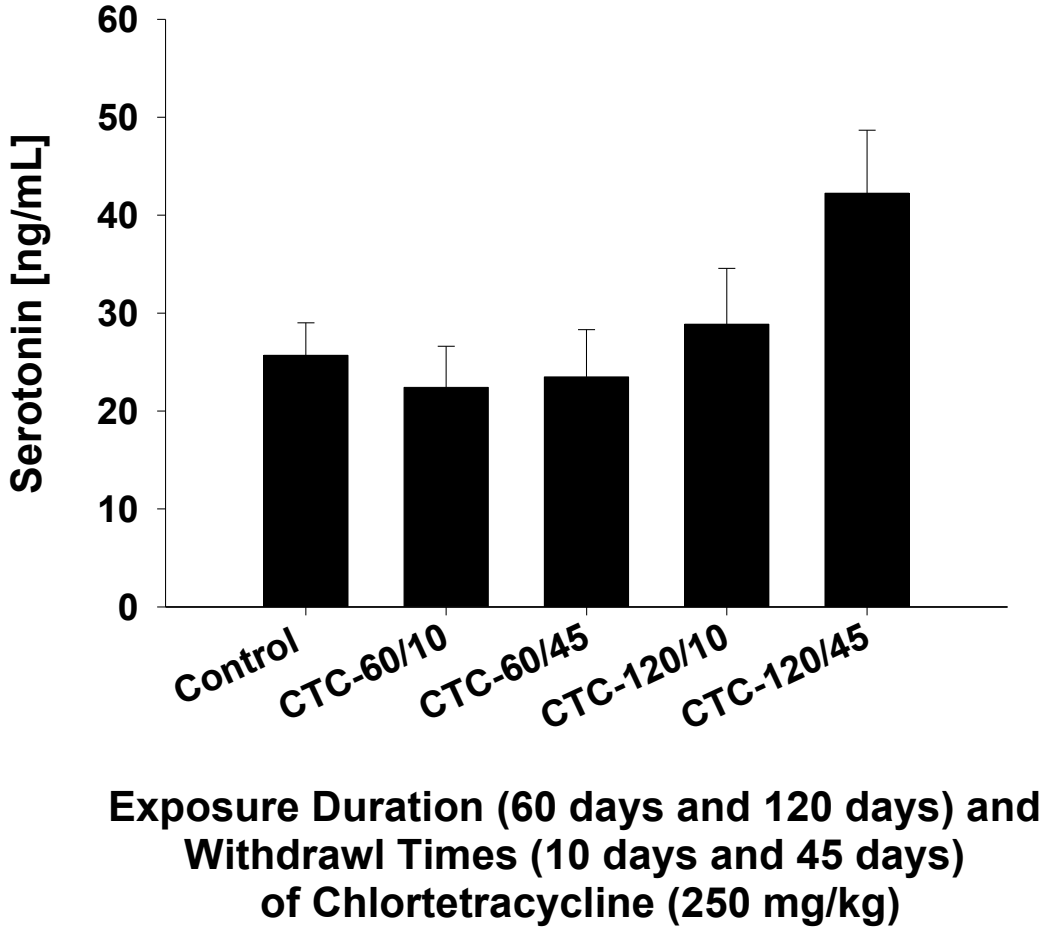

Figure 3.5 A Comparison of Female White-tailed Deer Serotonin Levels in Serum Following Exposure to Chlortetracycline in Feed and the Control Group

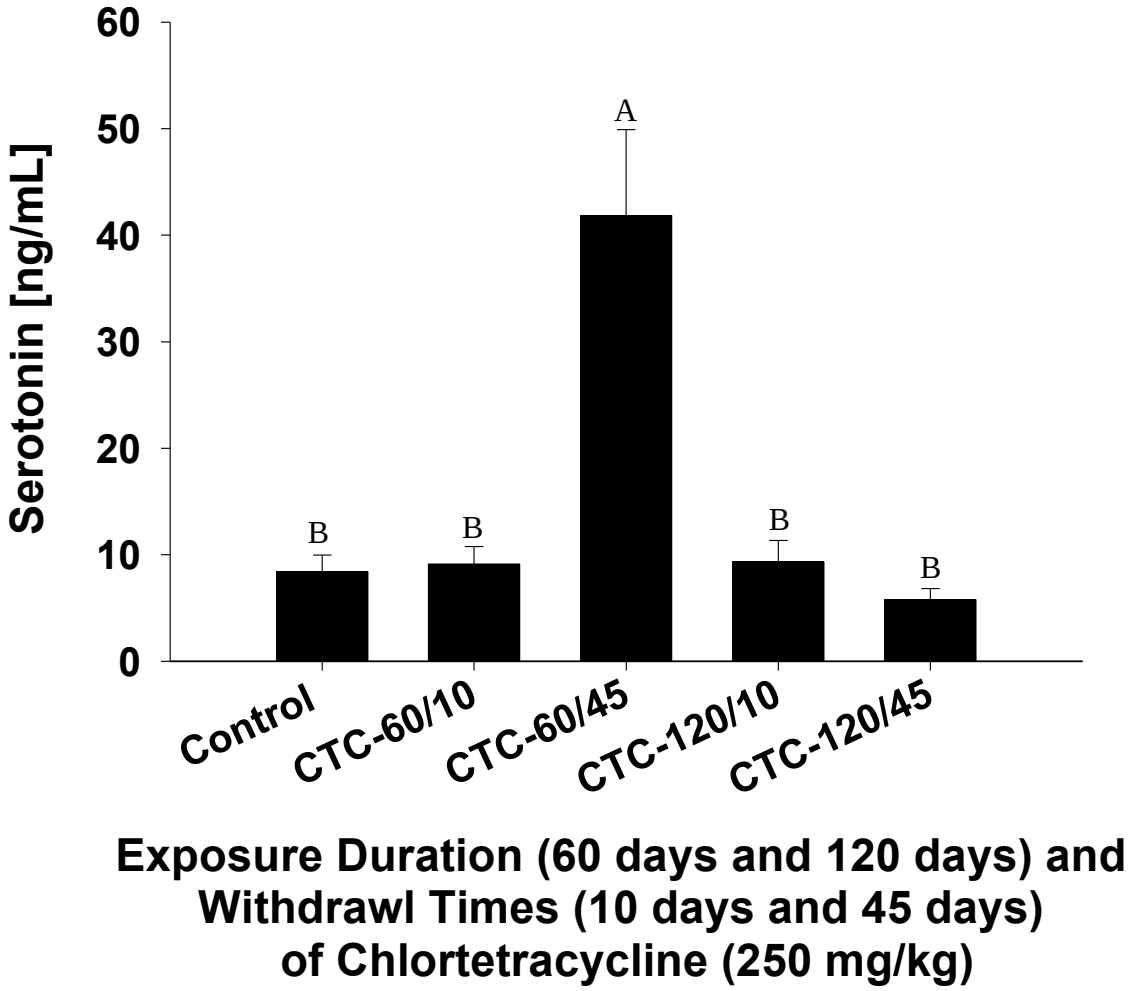

Figure 3.6 A Comparison of Female White-tailed Deer Serotonin Levels in Urine Following Exposure to Chlortetracycline in Feed and the Control Group

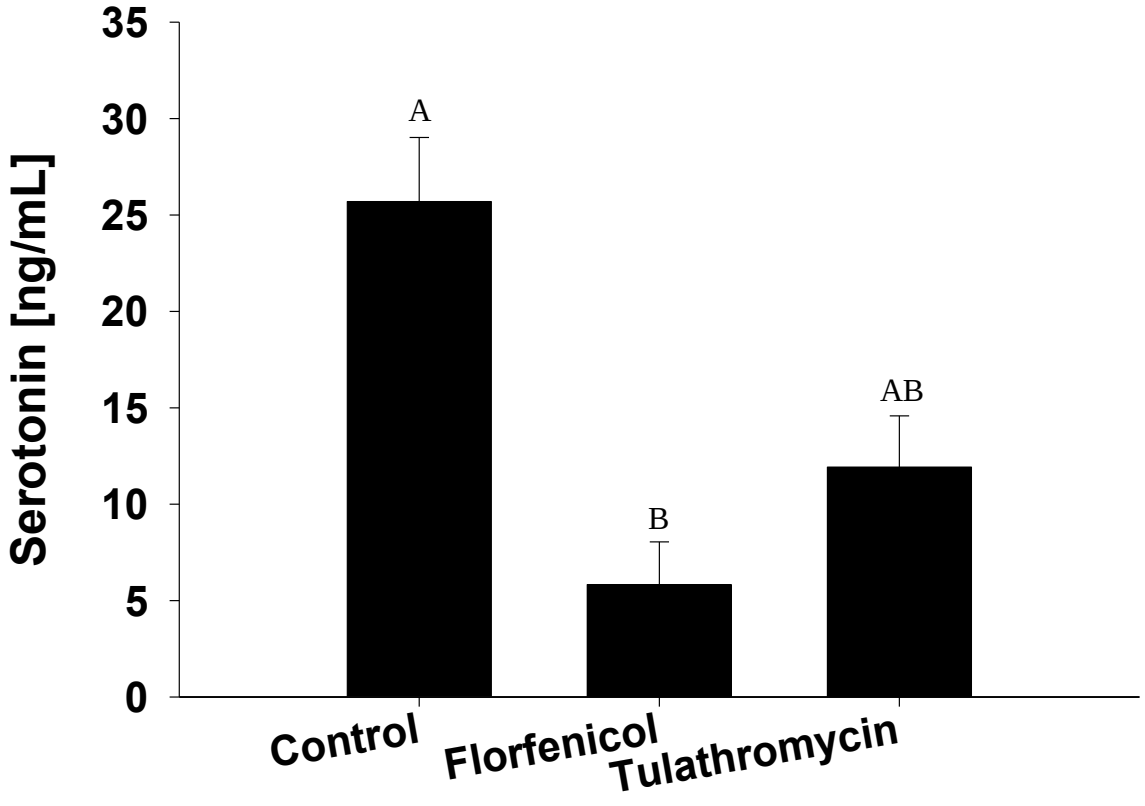

Figure 3.7 A Comparison of Female White-tailed Deer Serotonin Levels in Serum Following Exposure to Florfenicol, Tulathromycin and the Control Group

Conclusion

In summary, a quantitative liquid chromatography-mass spectrometry method was optimized for the detection and confirmation of 5-hydroxytryptamine (5-HT) in white-tailed tissues following exposure to antibiotics. While our results are not consistent with what previous studies have seen in terms of antibiotics affecting serotonin concentrations, it is important to note that those studies were conducted in monogastric animals. Whereas white-tailed deer are a ruminant species with a complex stomach. This may be why we are seeing different results. Further research needs to be done to increase the sample size of this antibiotic data and to capture a better idea of how antibiotics as AGPs are impacting serotonin concentrations in white-tailed deer.

CHAPTER 4

CHARACTERIZATION OF GUT MICROBIOTA FOLLOWING EXPOSURE TO ANTIBIOTICS IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)

Abstract

The work presented in this study provides insight into the bacterial populations in the gastrointestinal tract of white-tailed deer. External fecal samples were collected from treated and control deer pens. Internal fecal and rumen samples were collected from three control and three treated deer following euthanasia. The treated group received sixty days of chlortetracycline (CTC) treated feed containing 250 mg/kg of CTC. After sixty days, the treated group received control feed without CTC for the duration of the withdrawal period which was ten days. Next-generation sequencing of 16S rRNA was used to characterize the bacterial communities in the external and internal fecal samples as well as rumen samples of white-tailed deer. Bacteroidetes and Firmicutes were the dominant bacterial phyla in all cases. Changes in the microbial community that was observed between control and treated samples included a consistent increase in the abundance of Firmicutes and a decrease in the abundance of Bacteroidetes in all groups. In summary, we characterized the bacterial phyla in captive white-tailed deer fecal and rumen samples following exposure to chlortetracycline. Exposure to CTC leads to consistent increases in the abundance of the Firmicute phyla and consistent decreases in the abundance of the Bacteroidetes phyla.

Introduction

Like any other ruminant species, the white-tailed deer gut microbiome is composed of diverse and complex microbial communities (archaea, bacteria, protozoa, fungi, and bacteriophages) and is characterized by its diversity, high density and complex interaction [95]. The health and production of ruminants are highly dependent on the rumen microbial community which is highly responsive to dietary changes [38, 96]. Thus, the diet structure is the most significant factor of the hierarchical structural changes in bacterial communities in healthy growing ruminants

[39]. Other factors affecting the gut microbiome composition include genetics, environmental influence, host health and immune function, neurotransmitters and metabolites secreted by the host [54]. The gut microbial community supports the host in the extraction of nutrients, immune system and epithelium growth, and protects the host against pathogens [95]. Chlortetracycline is commonly used in the white-tailed deer industry as an antibiotic growth promoter to improve production and animal health [35]. The purpose of this study is to investigate the changes of the white-tailed deer gastrointestinal microbiota after an exposure of 60 days of in-feed chlortetracycline at a sub-therapeutic dose.

Materials and Methods

Animal Care and Treatment: Chlortetracycline

This experiment was performed in compliance with the appropriate laws and institutional guidelines approved by the Texas Tech Institutional Animal Care and Use Committee, protocol number 14039-08. The feed for this experiment was prepared by TTU feed mill, New Deal, TX.

A single batch of feed was prepared for each group. The control feed was prepared by pelleting a homogeneous mixture of cotton seed, alfalfa meal, corn grain, molasses, ammonium chloride, tallow, and sheep supplement premix. The CTC-treated feed was prepared by adding chlortetracycline to the mix to give a target concentration of 250 mg CTC/kg feed. Representative samples were taken from 5 bags of each of the experimental feed to analyze the CTC concentration. The samples were stored at -80°C until they were analyzed.

Eleven healthy white-tailed does with ages ranging from 2 to 6 years old and weighing between 35 and 49 kg were randomly divided into 2 groups: one control group and one treated group. Both groups were kept in high fenced pens. They were given *ad libitum* access to feed and water throughout the experiment. The treated group consisted of six animals that received sixty days of chlortetracycline treated feed containing 250 mg/kg of CTC. The control group consisted of five animals that received feed without CTC. After sixty days, the treated group received control feed

for the duration of the withdrawal period which was ten days. Lab personnel, as well as the university's veterinarians, monitored the health of the animals.

Sample Collection and Preparation

External fecal samples were collected from both the treatment and control group pens throughout the study and stored at -80℃ until analysis. Samples of rumen contents and colon fecal samples were removed immediately after euthanasia on day ten of feed withdrawal. The rumen samples collected were both solid and liquid digesta. The samples were then immediately stored in a container with ice in the field until transported to the laboratory where it was then stored at -80℃ until analysis. DNA extraction was done according to the instruction of the kit, total genome DNA from fecal and rumen samples were extracted using a QIAamp DNA Microbiome Kit (Qiagen, U.S.A.).

16S rRNA Sequencing

The 16s rRNA gene was amplified using the universal 16S amplicon PCR primers: forward primer 16S rRNA gene was amplified using the 515 Forward (5'-GTGCCAGCMGCCGCGGTAA-3') and 806 Reverse (5'-GGACTACHVGGGTWTCTAAT-3'), primers by Research and Testing Laboratory, Lubbock, Texas (RTL, Lubbock, TX, USA) [97].

Sequence Processing and Analysis

All sequencing reads were provided by the Research and Testing Laboratory's standard microbial analysis pipeline. In summary, the two major stages data analysis pipeline is comprised of, the denoising and chimera detection stage and the microbial diversity analysis stage. Throughout the denoising and chimera detection stage, denoising was achieved by utilizing several techniques to remove short sequences, singleton sequences, and noisy reads using the USEARCH and UPARSE algorithms [98, 99]. When the low-quality reads are removed, chimera detection was done to assist in the removal of chimeric sequences using the UCHIME chimera detection software in de novo mode [100]. Finally, the remaining sequences were then corrected per-base to help remove errors in sequencing. Sequences were then assigned into the

same operational taxonomic unit (OTU) at 97% identity using the UPARSE algorithm [99].

Results and Discussion

Overview of the Bacterial Composition and Relative Abundance

In this study, 16S sequencing of the rumen and fecal microbiome of antibiotic-treated and untreated feed fed to white-tailed deer was utilized to determine the effect of the antibiotic on the microbiome. Preliminary data for the diversity and richness of the microbial community in the rumen, cecum and external fecal samples were obtained from control (no antibiotic) and treated (provided antibiotic feed) animals.

A total of 380,270 high-quality reads were obtained from 4 fecal and 2 rumen samples of white-tailed deer and classified into operational taxonomic units (OTUs). While the majority of the OTUs were identified at the genus (g.) or species levels, some were only classified at the phylum (p.), class (c.), order (o.), or family (f.) level. For the purpose of this study, we are looking at the broad overview of the white-tailed deer gastrointestinal microbiome and will only be discussing changes between the domain and phyla of the control and treatment groups.

Tables 4.1-4.3 represent the relative abundance of the samples at the domain level for fecal and rumen samples. The majority were the bacteria domain. In fecal samples, the relative abundance ranged from 91 to 96% bacteria while the rumen ranged from 83 to 89% relative abundance. There were "No Hit – Unknown" results as well. In fecal samples the abundance of "No Hit – Unknown" ranged from 3 to 9% while it ranged from 10 to 17% in the rumen samples. There was also a small percentage of the archaea domain. In fecal samples, the percent relative abundance ranged from 0.2 to 2% while it was constant at 0.4% in rumen samples. Research has found that bacteria makes up between 40 and 90% of total microbial mass while *Methanogens* only make up between 2 and 4% [36]. Our study shows that the bacteria found in white-tailed deer rumen accounts for 83 to 89% of microbial mass which falls in line with what is expected based on literature, but our study only found 0.4% Archaea (*Methanogens*) which is lower than literature reports. This may be that some

Methanogens were "Unknown" and came back as "No Hit" during the microbial analysis. Genetics, diet, environmental influence, host health, immune functions, neurotransmitters and metabolites secreted by the host are also factors that may affect the gut microbiome [54].

Table 4.1 Percent Relative Abundance of the External Fecal Samples at the Domain Level

Domain	Control	Treated	Change Relative to Controls
Bacteria	94	92	2% Lower
Archaea	0.2	3	1.8% Higher
Not Hit – Unknown	6	5	1% Lower

Table 4.2 Percent Relative Abundance of the Internal Fecal Samples at the Domain Level

Domain	Control	Treated	Change Relative to Controls
Bacteria	91	96	5% Higher
Archaea	0.2	0.5	0.3% Higher
Not Hit – Unknown	9	3	6% Lower

Table 4.3 Percent Relative Abundance of the Rumen Samples at the Domain Level

Domain	Control	Treated	Change Relative to Controls
Bacteria	89	83	6% Lower
Archaea	0.4	0.4	No Change
Not Hit – Unknown	10	17	7% Higher

Tables 4.4-4.6 show the relative abundance of the top phyla per treatment group and how the relative abundances changed with antibiotic treatment. There were notable changes in the phyla level distribution between control and treated samples. The Firmicutes was most abundant in all cases with the exception of the rumen control samples. Bacteroidetes was the second most abundant phylum in all groups. Changes in the microbial community that was observed between control and treated samples included a consistent increase in the abundance of Firmicutes and a decrease in the abundance of Bacteroidetes in all groups. This is consistent with other studies that

identify Firmicutes and Bacteroidetes as dominant phyla in ruminants [47, 50]. The other phyla found in both the external and internal fecal samples include Spirochaetes, Verrucomicrobia, Proteobacteria, Lentispaeria, Actinobacteria and Tenericutes. The internal fecal samples also differed from the external samples in that they had low levels of *Fusobacteria* in the control but not in the treated internal fecal samples. They also had low levels of Fibrobacteres in the treated internal fecal samples but not the control samples.

The other rumen phyla differed slightly and include: Spirochaetes, Proteobacteria, Actinobacteria, Tenericutes, and Chloroflexi. The rumen samples did not contain Lentispaeria as the fecal samples did. It is also interesting to note that the rumen control samples contained a low level of *Fretibacterium sp.* of the phylum Synergistetes, but the treated rumen samples did not detect it.

Table 4.4 Percent Relative Abundance of External Fecal Samples at the Bacterial Phyla Level

Phyla	Control	Treated	Change Relative to Controls
Firmicutes	45	49	4% Higher
Bacteroidetes	35	22	13% Lower
Spirochaetes	0.7	4	3.3% Higher
Verrucomicrobia	0.7	0.8	0.1% Higher
Proteobacteria	1	0.7	0.3% Lower
Lentispaeria	0.3	0.9	0.6% Higher
Actinobacteria	0.07	0.06	0.01% Lower
Tenericutes	0.04	0.1	0.06% Higher
Fibrobacteres	Not Detected	2	2% Higher

Table 4.5 Percent Relative Abundance of Internal Fecal Samples at the Bacterial Phyla Level

Phyla	Control	Treated	Change Relative to Controls
Firmicutes	40	44	4% Higher
Bacteroidetes	32	18	14% Lower
Spirochaetes	3	13	10% Higher
Verrucomicrobia	4	2	2% Lower
Proteobacteria	0.5	3	2.5% Higher
Lentispaeria	0.08	0.03	0.05% Lower
Actinobacteria	0.09	0.1	0.01% Higher
Tenericutes	0.03	0.1	0.7% Higher
Fibrobacteres	Not Detected	0.5	0.5% Higher
Fusobacteria	0.02	Not Detected	

Table 4.6 Percent Relative Abundance of Rumen Samples at the Bacterial Phyla Level

Phyla	Control	Treated	Change Relative to Controls
Firmicutes	14	20	6% Higher
Bacteroidetes	21	9	12% Lower
Spirochaetes	1	1	No Change
Verrucomicrobia	0.07	Not Detected	
Proteobacteria	4	6	2% Higher
Actinobacteria	0.06	0.1	0.04% Higher
Tenericutes	0.08	0.	0.08 % Lower
Chloroflexi	0.06	0.4	0.34 % Higher
Synergistetes	0.1	Not Detected	

Table 4.7 shows the archaea phyla percent relative abundance and the percent change observed between the control and treatment groups. There was only one phylum found and it was *Euryarchaeota*. The primary class in that phylum being Methanobacteriaceae. For the most part, the relative abundance between groups remained constant with exception of the external fecal samples. In the external fecal samples, the relative abundance increased by 0.01% when treated with CTC.

Table 4.7 Percent Relative Abundance of the Fecal and Rumen Samples at the Archaea Phyla Level

Tissue	Phyla	Control	Treated	Change Relative to Controls
External Fecal	Euryarchaeota	0.1	0.2	0.1% Higher
Internal Fecal	Euryarchaeota	0.1	0.1	No Change
Rumen	Euryarchaeota	0.4	0.4	No Change

Research shows that plant-based diets are predominated by Bacteroidetes and Firmicutes [54, 57, 58]. The feed provided to the white-tailed deer in this study were plant-based and the predominant bacterial phyla were Bacteroidetes and Firmicutes. It has been determined that the dominant bacterial phyla in wild Canadian cervids are Bacteroidetes and Firmicutes which is supported by the data from this study. But the relative abundance of each varies among ruminant species and also between phases of rumen digesta such as solid and liquid digesta [50]. Gruninger et al., determined that Firmicutes were more prevalent in solid rumen elk samples and Bacteroidetes was found at a high prevalence in the liquid rumen samples [50]. While the bacterial composition found in solid and liquid rumen digesta samples of the rumen were distinctly different in elk, it was not the case for deer. In our study, the samples collected and analyzed were a mixture of solid and liquid digesta for the rumen samples of white-tailed deer.

A study by Ishaq and Wright characterized bacterial communities in free-ranging moose from Vermont, Alaska, and Norway. They found that the Bacteroidetes phylum was most abundant in moose from Alaska and Norway, but the Firmicutes phylum was more abundant in moose from Vermont. In the samples from Alaska, Firmicutes was the third most abundant phylum and in the Norway samples, it was the second most abundant phylum. It seems location was a strong factor in shaping the gut microbiome [47]. Researchers have found Bacteroidetes was the dominant phyla in ruminants on a high starch diet whereas Firmicutes were dominant in ruminants on a high fiber diet [47, 78, 79]. Our study determined Firmicutes was the dominant phyla in white-tailed deer with the exception of the rumen control samples. This could be for many reasons such as location, diet, and ruminant species. The white-tailed deer

samples in our study were from a captive facility in Texas. Where are there studies mentioned above were a different ruminant species and were free-ranging animals which means their diet differed significantly from that of our deer.

In our study, the Fibrobacteres phyla were detected in the external, 2%, and internal, 0.5%, treated samples but not the control samples. It was found that the abundance of Fibrobacter phyla varies among elk solids and liquids, but very low levels were found in deer samples. The main role of Fibrobacter is to aid fiber digestion in some ruminant species [50]. It is interesting that our study only detected the Fibrobacteres phyla in the treated fecal samples. One of the most prevalent bacterial species in the rumen is *Fibrobacter succinogenes* [36]. But *Fibrobacter succinogenes* was not detected in either rumen sample.

Conclusion

The ruminant microbiome is one of the most well studied microbial ecosystems. In this study, the changes of in-feed chlortetracycline on the white-tailed deer gastrointestinal microbiota was assessed by analyzing preliminary data following 60-day exposure to the antibiotic and a 10-day withdrawal period. The results from this preliminary data indicate that low, short-term dose of AGP increased the Firmicute phylum and reduced the Bacteroidetes phylum in the white-tailed deer gut microbiota. Further examination is needed to conclude whether the treatment of chlortetracycline at sub-therapeutic doses is helping to suppress potentially harmful bacteria and maintain the potentially beneficial bacteria.

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