IDENTIFICATION AND METABOLISM OF SUVOREXANT: IMPLICATIONS FOR FORENSIC TOXICOLOGY

_____

A Dissertation

Presented to

The Faculty of the Department of Forensic Science

Sam Houston State University

_____

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

_____

by

Britni N. Skillman

December 2019

IDENTIFICATION AND METABOLISM OF SUVOREXANT: IMPLICATIONS FOR FORENSIC TOXICOLOGY

by

Britni N. Skillman

_____

APPROVED:

Sarah Kerrigan, PhD
Committee Director

Madeleine Swortwood, PhD
Committee Member

Jorn Yu, PhD
Committee Member

Donovan Haines, PhD
Committee Member

Phillip Lyons, PhD
Dean, College of Criminal Justice

DEDICATION

"Wit beyond measure is man's greatest treasure."
-J.K. Rowling, Harry Potter and the Order of the Phoenix

To my advisor, Dr. Kerrigan, for all the late nights, weekends, and holidays sacrificed to ensure that I had every resource I needed for this journey, and for her undying belief in me even in times I struggled to believe in myself.

To my family for their constant encouragement and support along the way and to my mom for being an unrivaled role model.

To my peers for their reassurance, laughter, and great company.

To my cat, Frosty, for surviving with me all the way from junior high school to the end of my PhD.

"Do or do not. There is no try."
-Yoda, Star Wars: The Empire Strikes Back

ABSTRACT

Skillman, Britni N., *Identification and metabolism of suvorexant: Implications for forensic toxicology*. Doctorate of Philosophy, Forensic Science, December, 2019, Sam Houston State University, Huntsville, Texas.

Suvorexant (Belsomra®), a novel dual orexin receptor antagonist for the treatment of insomnia, was recently introduced to the pharmaceutical market in 2015. Insomnia affects up to one-third of the American population, which could make suvorexant a popular option for treating these patients. However, due to its recent introduction to the market, few methods have been developed for the detection of suvorexant and limited case reports have been published that examine suvorexant in forensic toxicology casework. Since a limited number of studies exist detailing the analysis of suvorexant, little is known regarding its role in human performance toxicology and postmortem investigations. This study aimed to further the understanding related to its analytical detection, the identification of metabolites, and the drug's physicochemical properties. In broader terms, the potential for drug-mediated interferences using liquid chromatography-mass spectrometry (LC-MS) is also addressed.

Methods for the detection of suvorexant in blood at forensically relevant concentrations were developed and validated using liquid chromatography-quadrupole/time-of-flight-mass spectrometry (LC-Q/TOF-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Ion suppression and matrix effects using electrospray (ESI) techniques were evaluated and strategies for mitigating interferences in quantitative targeted assays were assessed. The importance of using stable isotope labeled internal standards (SIL-IS) was highlighted using a statistical comparative approach with a structurally similar analog. Suvorexant was quantitated in forensic case specimens and

its lipophilicity was determined experimentally and theoretically to evaluate its potential to undergo postmortem redistribution (PMR). In the absence of commercially available metabolite standards, major metabolites for suvorexant were produced *in vitro* using recombinant cytochrome P450 enzyme systems and were subsequently identified in authentic case specimens.

KEY WORDS: Suvorexant, High resolution mass spectrometry, LC-Q/TOF-MS, LC-MS/MS, Ion suppression, Matrix effects, Blood, Forensic toxicology, Metabolism, Postmortem redistribution

ACKNOWLEDGEMENTS

We would like to give special thanks to Dr. Barry Logan (National Medical Services Labs/Center for Forensic Science Research and Education) and Daniel Anderson, MS, (Colorado Bureau of Investigation) for providing adjudicated forensic specimens for analysis.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Insomnia

Insomnia is a prevalent medical condition that affects approximately one-third of the adult population (1, 2). Of Americans suffering from the condition, one-half reported having at least one symptom of insomnia per night and one-third reported having insomnia every night (3). The consequences of insomnia that affect daytime activities have included irritability, inability to concentrate, low energy levels, absence from work, and poor job performance. In addition, insomnia can increase risk for traffic-related accidents and can lead to other health problems (3). Direct and indirect costs related to insomnia are estimated to exceed $100 billion per year in the United States, and the US market for insomnia medications had already been projected to exceed $5 billion by the year 2010 (3). Persons with underlying medical conditions such as gastrointestinal problems, hypertension, pulmonary diseases, or urinary problems may experience higher levels of insomnia than the general population (1). Individuals suffering from insomnia are significantly more likely to suffer from depression and anxiety, and sleep disorders are part of diagnostic criteria for a number of psychiatric disorders including bipolar disorder, post-traumatic stress disorder, and major depressive disorder (1). It may also serve as a risk factor for substance abuse and an overall diminished quality of life (4). Primary insomnia sufferers may have difficulties in initiating or maintaining sleep, experience premature awakening, or may find that their sleep is non-restorative (5, 6). Treating insomnia has become a concern of socioeconomic interest as well as improving the quality of life of those individuals who experience its symptoms.

Finding safe treatments for insomnia that improve sleep while avoiding dependence, next-day impairment, and rebound insomnia has been an ongoing and challenging task (3). Typically, the aim of insomnia medications is to promote sleep, maintain sleep architecture, and to avoid association with residual side effects (7). The ideal insomnia drug is dependent on many pharmacokinetic and pharmacodynamic parameters, receptor binding, potency, and mechanism of action (7). Timing of receptor activity is key with the treatment of insomnia, as a rapid central nervous system penetration is desired to increase sleep-onset efficacy, and the receptor occupancy must be sufficiently high to maintain sleep; however, the compound occupancy at the receptor must drop before the desired time of wakening (7). For example, a compound that is effective at low receptor occupancies may promote sleep onset more efficiently, but the likelihood of residual effects is greater, while a compound with high occupancy threshold for sleep-promoting effects may have less inherit risk for carry-over effects (7).

Several therapeutics have been developed that have aimed to achieve the desired effects while minimizing side effects, but the perfect insomnia drug still eludes pharmaceutical companies. Traditional treatments for insomnia have included histamine receptor agonists, γ-aminobutyric acid (GABA) receptor agonists, and melatonin receptor agonists (6, 8, 9). Most widely prescribed are central nervous system depressants that directly act on GABA which is an inhibitory neurotransmitter. Sedative hypnotics have typically included drugs such as barbiturates, benzodiazepines, and non-benzodiazepines that modulate the activity of GABA-A receptors (9). Barbiturates were used for the treatment of insomnia beginning in the early 1900s, but led to abuse, physical dependence, withdrawal, and overdoses in large part due to respiratory depression. Treatments then

shifted to benzodiazepines in search of a barbiturate alternative (10). Benzodiazepines have been commonly used as GABA modulators since their development in the early 1960s, but are associated with dependence risks, impaired memory, and daytime sleepiness (3). Some benzodiazepines that have been used in the treatment of insomnia are estazolam, flurazepam, lorazepam, quazepam, temazepam, and triazolam (11). Non-benzodiazepine GABA modulators have been developed more recently, including the "Z-drugs" (zolpidem, zopiclone/eszopiclone and zaleplon), but there are safety concerns with these sedative hypnotics as well (3). Both benzodiazepine and non-benzodiazepine GABA-A receptor modulators have an increased risk for side effects such as next-morning sedation and cognitive residual effects, which is in part due to the long half-lives of some of these treatment options (7). Moreover, these GABA-A modulators likely are associated with residual activity during the wake period due to their low receptor occupancy threshold for efficacy *in vivo* (~27%) (7). A melatonin receptor agonist for the treatment of insomnia is ramelteon which is novel in its approach, but also poses concerns for safety and efficacy (3). Sedating and tricyclic antidepressants have also been prescribed for insomnia treatment, such as doxepin, amitriptyline, mirtazapine, and trazadone which was the most commonly prescribed insomnia medication in 2002 (6, 11). Anticonvulsants such as gabapentin and pregabalin have been used, as well as antipsychotics like olanzapine and quetiapine (6). The most recent approach to insomnia treatment has been the modulation of the orexin signaling system. Since reduction in the function of the orexin signaling system leads to a decrease in wakefulness, antagonists of the orexin system have become a promising approach to treating insomnia as well as other disorders that interrupt the circadian rhythm, such as jet lag or shift work (3, 12).

Orexin Receptor Antagonism

Orexin (hypocretin) neuropeptides A and B were only recently discovered in 1998, which paved the way for orexin receptor 1 and 2 (OX_1R and OX_2R) characterization and drug development for orexin receptor antagonism (3, 5, 12). Both OX_1R and OX_2R are G-protein coupled receptors (9, 13). Orexin A binding affinity to OX_1R is greater than that of orexin B, but binding affinity to OX_2R is equal for both orexin A and B (3, 14). The peptide orexin is produced in the perifornical area/latero-posterior hypothalamus by about 50,000-80,000 neurons in humans. From the lateral hypothalamus, the projections reach areas of the neuraxis to include the tuberomammillary nucleus (TMN), laterodorsal tegmental nucleus (LTD), paraventricular thalamic nucleus (PVT), and arcuate nucleus of the hypothalamus (5, 14, 15). Less dense projections are present in the amygdala, hippocampus, and colliculi (5). The production of orexin can result in reduced activity of parts of the brain that promote sleep, thereby increasing wakefulness. It is hypothesized that orexin plays a large role in the sleep to wake cycle, and release of the neuropeptide typically follows the circadian rhythm (1, 10, 12, 16). Orexin neurons primarily fire during waking and activate wake state-favoring centers such as the locus coeruleus and dorsal raphe, and firing stops when sleep begins (5, 12, 14, 17). Shortly after this discovery, defects in the orexin system at the peptide level in dogs were related to narcolepsy/cataplexy, and it was found that orexin producing cells were largely absent in the lateral hypothalamus (5). Orexin was found to be a predominating mediator of arousal after increased arousal was observed following exogenous administration of orexin-A to animal models with narcolepsy (10). Narcolepsy is a condition characterized by instability of the wake cycle which results in symptoms such as excessive daytime sleepiness and

daytime hypersomnia (12, 18). The role of orexin peptides in narcolepsy was first described in animal models which helped to establish the link between the disorder and orexin dysfunction (15, 18, 19). After discovery that mutation in the orexin 2 receptor in dogs was very similar to that of humans, it was suggested that the loss of signaling that is mediated by this receptor could be responsible for narcoleptic phenotypes in humans as well, and the disorder is more prevalent in familial clusters (18). Narcoleptic patients are at a three-times higher risk to be involved in motor vehicle accidents due to lack of alertness and dozing off (18). Narcolepsy has also been associated with sleep fragmentation, premature awakenings, vivid dreams, hypnagogic hallucinations, and sleep paralysis during nocturnal sleep (18). Patients can suffer with narcolepsy with or without cataplexy, which is a sudden episode of muscle weakness during consciousness that is usually triggered by a strong emotional stimulus (5, 18). About 0.05% of the population is affected by narcolepsy with cataplexy (5). The lack of orexin producing neurons in the lateral hypothalamus has been documented in postmortem studies of individuals suffering from narcolepsy, and patients with the condition have been reported to have the absence or very low levels of orexin in the cerebrospinal fluid (5, 15). The discovery of the relationship between orexin signaling and narcolepsy has given scientists a new way to try to mitigate the effects of insomnia, by mimicking the effects of narcolepsy seen due to orexin deficiency. The therapeutic potential of selective or dual orexin receptor antagonists has been investigated in an attempt to target both receptors in the function of the sleep/wake cycle but without causing cataplexy.

As such, a new class of medications called dual orexin receptor antagonists (DORAs) has proved a promising alternative approach to treating insomnia since they have

no effect on GABA activity (7). It has been reported that many orexin receptors are glutaminergic, but they are not GABAnergic (5). DORAs inhibit orexin neuropeptides and their wake-promoting activities, as opposed to promoting sleep itself (7). Higher percentages of receptor occupancy are required for their efficacy and to block the effects of the orexin peptide ligands (7). Many pharmacodynamic factors are responsible for the effects of insomnia medications in addition to plasma half-lives, such as the mechanism of action which can dictate a drug's receptor occupancy levels in order to be effective. As such, the need for high effective receptor occupancy in the use of DORAs can allow them to promote sleep at lower doses (12). These DORAs are suspected to have less "hangover" effects than benzodiazepines and Z-drugs because they do not suppress rapid eye movement (REM) sleep and do not affect memory as GABA-A modulators might (5). Studies on the mechanisms of orexin receptor antagonists have shown that response to arousal stimuli is preserved with the use of these agents, in contrast to zolpidem and eszopiclone which impair ability to arouse to salient stimuli (10, 12). Arousability is necessary for normal psychological responses during sleep, and it seems that DORAs can preserve the arousal threshold, while the threshold is increased with GABA-enhancing drugs (12). Since OX_2R seems to have more implications in sleep/wake regulation than OX_1R, the possibility of using single orexin receptor antagonists (SORAs) is being explored (12). The pharmacokinetics and receptor-binding kinetics of DORAs make them promising candidates in restricting their effects to the resting phase (7). Critical requirements of any DORA in development are that they should have a rapid onset of action, a duration of action lasting no more than 8 hours, a short-half life, and they should avoid accumulation at the receptor site (5, 15, 20). Some advantages in the early stages of

development in the first DORAs are the absence of anterograde amnesia that is commonly seen with other narcoleptics and perhaps a lower abuse potential (5).

The use of DORAs has also been explored to characterize their potential use in treating depression, anxiety, pain conditions, and neurogenerative disorders such as Alzheimer's disease (10, 12, 20). It has also been noted that since sleep and migraines are interconnected, that the regulation of sleep through the use of DORAs might provide a novel approach to migraine prevention (12). These compounds may also provide an alternative to hypnotics given to patients experiencing lack of sleep due to post-operative pain, and can reduce the occurrence of post-operative delirium as a result (12). Orexin modulation may also have a role in regulating rewarding and reinforcing properties of drugs of abuse, and preliminary research has shown that DORAs can reliably reduce cocaine's rewarding properties in clinical trials (17). It has been suggested that orexin transmission to and within the ventral tegmental area (VTA) in the brain is highly implicated in the reinforcing effects of cocaine and morphine (12, 13). Studies are ongoing to assess the diminishing effects of drug-induced mesolimbic dopamine transmission from the VTA following blockade of orexin transmission (17). Orexin receptor targeting may provide a new approach to treating opioid use disorder as well (13). Research has shown that the lateral hypothalamus plays a large role in drug-seeking and reward behavior, and that orexin may be responsible for linking the lateral hypothalamus and mesolimbic pathway in the processing of reward/reinforcement (13).

There have only been a handful of DORAs developed to date and only one is currently on the market. Almorexant was the first DORA that was characterized in clinical trials for the treatment of insomnia by Actelion Pharmaceuticals in Switzerland. It made it

to phase II clinical studies, however it did not meet registration stages of approval (5). It was shown that almorexant was associated with infrequent transient increases in liver enzymes, which were found to be unrelated to the orexin system (12, 20). GlaxoSmithKline (GSK) also developed a DORA known as SB-649868 (or GW-649868) but like almorexant, it failed prior to phase III clinical trials and is no longer in development (5, 12). Suvorexant was the first DORA to reach the registration stage with the FDA, and subsequently became commercially available (5, 8). It is also the first new-class insomnia medication to be introduced to the market since ramelteon (Rozerem®) was approved for use in 2005 (11). Filorexant was a subsequent drug similar to suvorexant in its receptor binding and was being developed by Merck & Co., but is no longer listed in the production pipeline (5). Filorexant had a shorter half-life than suvorexant, but it was found to have increased next-day somnolence in a dose-dependent manner (12, 20). Lemborexant (E2006) is the newest proposed DORA which is currently in phase III clinical trials, but initial reports show that it provides greater efficacy at even lower doses and may minimize next-day somnolence (10, 12, 21). Seltorexant (JNJ-42847922) is the only SORA in development and in early clinical stages thus far (10, 12). Other SORAs previously explored were MK-1064 and MK-3697 (10). These DORAs and SORAs are depicted in **Figure 1.1**. From the reports, almorexant, suvorexant, filorexant, and SB-649868 have narcoleptic effects that reduce awakenings, lessen the time to sleep onset, and increase total sleep time (5). Suvorexant is the first drug in its class that has been prescribed to patients for the treatment of insomnia and is the topic of this report.

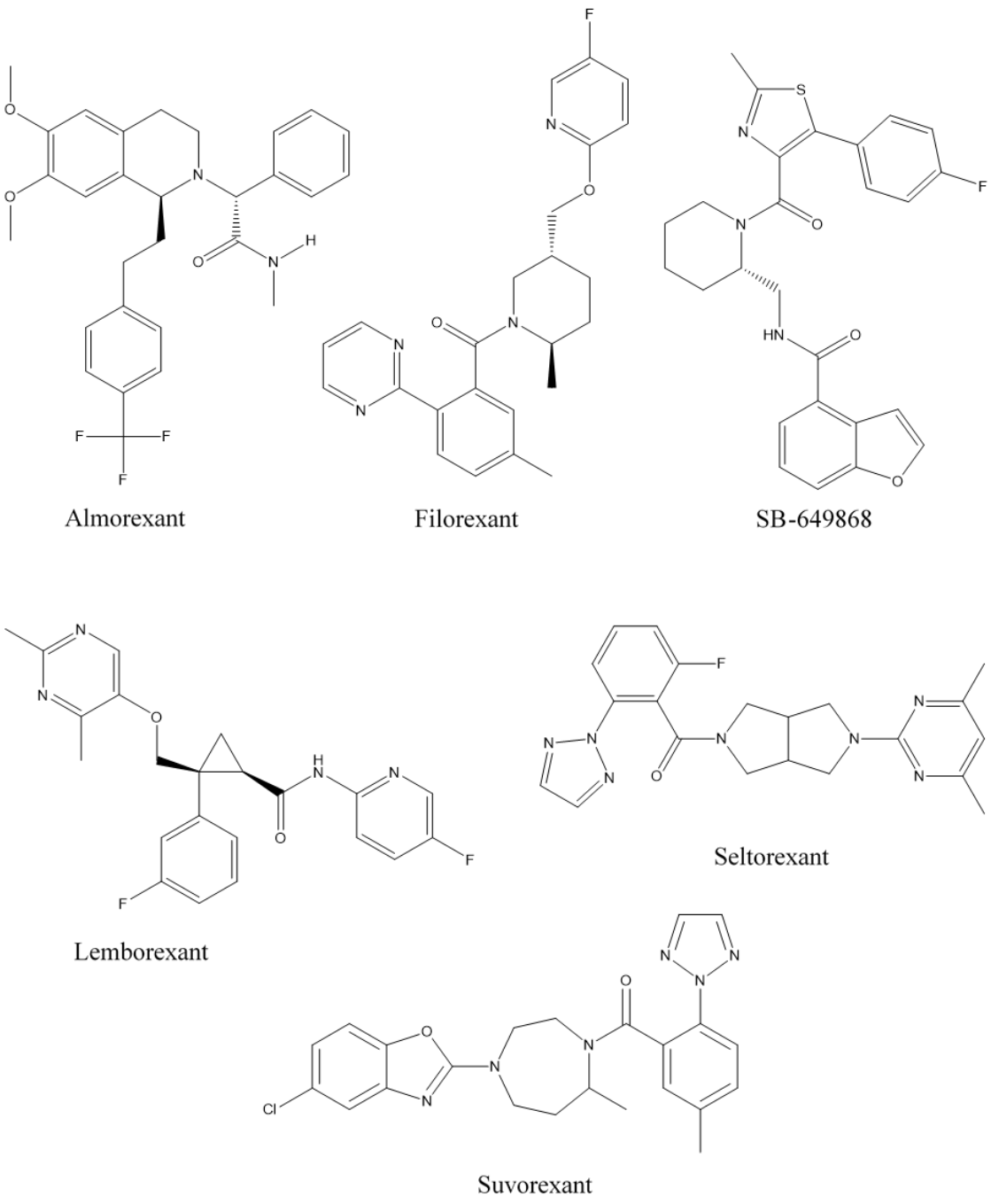


Figure 1.1. Chemical structures of DORAs (almorexant, SB-649868, filorexant, suvorexant, and lemborexant) and SORAs (seltorexant) that have been developed for the treatment of insomnia.

Suvorexant Background

Suvorexant (MK 4305) is a DORA currently available on the market in the United States and Japan (9, 12, 22, 23). It is manufactured by Merck & Co. and is marketed under the trade name Belsomra®. The chemical name for suvorexant is [(7R) -(4-(5-chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl] [5-methyl-2-(2H-1,2,3-triazol-2-yl) phenyl] methanone (24, 25). The suvorexant molecular formula is $C_{23}H_{23}ClN_6O_2$ with a molecular weight of 450.932 g/mol (4, 25). Suvorexant synthesis has been described by a few authors, but Cox et al. described the synthesis of suvorexant from a core diazepane ring, in which they noted that the diazepane core is key for the drug's potency (**Figure 1.2**) (3, 26-28). The chlorobenzoxazole has demonstrated improved metabolic stability, favorable target potency, and brain penetration over other DORA alternatives that preceded suvorexant in development (10).

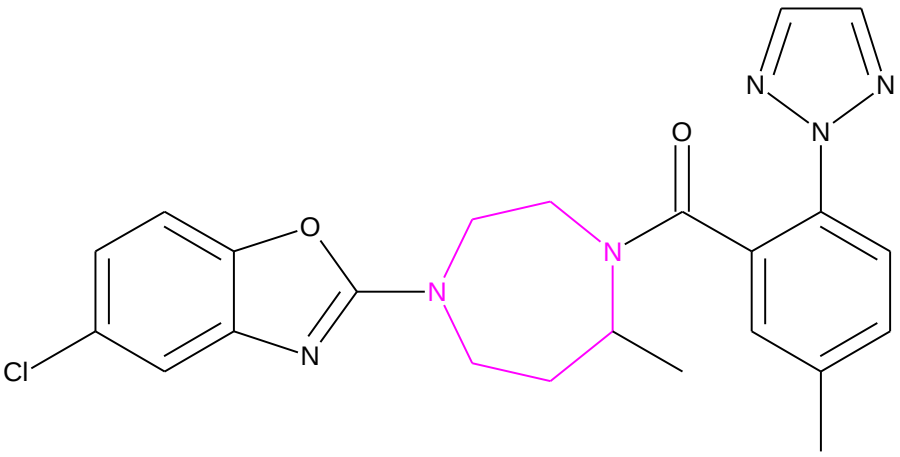


Figure 1.2. The structure of suvorexant depicting its core diazepane functionality responsible for the drug's potency.

Suvorexant was first approved for use in the United States in 2014 by the FDA and it is currently placed under Schedule IV of the Controlled Substances Act (CSA) (24,29). It is classified as a sedative hypnotic due to these drugs having the common pharmacological property of sedative activity (9). Although sedative hypnotics have been used in the treatment of insomnia, these drugs are associated with an inherent risk for physical dependence and withdrawal, and some animal studies have shown a resulting self-administration indicating the possibility for physical dependence (9). Suvorexant improves sleep onset and maintenance and is believed to have less side effects and more favorable tolerability than other therapeutic options (4, 30). The main benefit that was identified during clinical studies is the low potential for addiction or dependence with suvorexant (4, 31). FDA evaluation of the drug found that sleep induction is dose-dependent and that suvorexant may be unsafe at the higher concentrations originally proposed by Merck (30-40 mg) and that doses should be limited to 10-15 mg (1, 5, 8, 32, 33). At higher doses there were reports of sleep paralysis and narcolepsy-like events (6). It has also been noted that suvorexant, like other sedative hypnotics, should be avoided in patients with severe hepatic impairment (4). While insomnia and mood disorders often have a high comorbidity, patients with depression or psychiatric disorders have been excluded from suvorexant clinical trials. As such, there is a possibility of worsening depression or suicidal ideation in these populations with the use of suvorexant, although post-marketing surveys have indicated that the safety profile matches that of the product's labelling (10, 12, 31). Suvorexant is contraindicated in patients with narcolepsy, since symptoms due to orexin deficiency in narcoleptic patients could be intensified with the use of an orexin antagonist (8).

During trials, onset of sleep occurred more rapidly in individuals who received the 40-mg dose, which occurred between 56- and 68-minutes following administration (4). Suvorexant has the potential to produce next-day drowsiness which can interfere with daily activities, and the effect is more pronounced at doses >40-mg and increase as the dose increases (4, 8). The original proposed dose of suvorexant was 40-mg but recommendations were made to reduce the daily recommended dose to 10-mg after these safety concerns were made (4, 8). Increases in suvorexant dosage are only suggested for patients who display tolerability to the lower dose with no side effects (4). Unlike GABA-modulating agents which are only recommended for short term or intermittent use, suvorexant can be used long-term and on a daily basis with no risk of physical dependence (4, 34). The abrupt cessation of suvorexant administration has not been associated with withdrawal or rebound insomnia at the available prescribed doses (1, 4, 11, 34). Suvorexant is prescribed to adults 18 years and older (4). The safety profile of suvorexant indicates that the treatment be used for individuals under the age of 65, although the >65 age group is most likely to seek treatment for sleep impairment (4). Individuals over the age of 65 were more sensitive to the adverse side effects of suvorexant in clinical trials, including a significant impairment of balance (4).

A one-year controlled safety and efficacy study of the use of suvorexant to treat insomnia was described by Michelson et al. The study demonstrated that suvorexant was well tolerated by insomnia patients, both male and female, and elderly and non-elderly across various populations (35). The most reported adverse effect was somnolence, which aligns with other studies (8, 35, 36). Suvorexant studies were performed to determine if administration negatively affected patients with chronic obstructive pulmonary disease

(COPD) (36). There is a concern for patients suffering with COPD or other respiratory disorders, such as obstructive sleep apnea, since comorbidity with insomnia is about 17% in these individuals (36, 37). As such, there is an associated risk of further respiratory depression in these patients that are prescribed GABA-modulating sedative hypnotics for the treatment of insomnia (36). It is thought that orexin may have a role in respiratory function since there are orexin neurons that project through the brain to some respiratory centers (37). There is limited literature on this theory and studies were performed to determine the relationship between orexin receptor antagonism and respiratory impairment (37). Uemura et al. studied the effects of suvorexant on respiratory function during sleep on healthy male and female subjects and determined there were no adverse effects in healthy subjects, then suggested performing studies with population groups with COPD or obstructive sleep apnea (37). A study by Sun et al. demonstrated that subjects with COPD or obstructive sleep apnea that were given twice the maximum FDA-approved dose (40-mg) were generally able to tolerate suvorexant with no meaningful effects of respiration (36, 38). Although suvorexant should be taken with caution in these patients, it seems to be more well-tolerated in subjects with compromised respiratory function than traditional benzodiazepine options (36, 38).

Suvorexant slowly equilibrates at OX_1R and OX_2R receptors but has a high selectivity for both (5, 9). Suvorexant binds to these receptors over 6000x more selectively than to over 170 other receptors and enzymes that were studied (24). In both orexin receptors, suvorexant binding occurs at the orthosteric location (10). Suvorexant promotes sleep by blocking the binding of orexin A and B neuropeptides and is reversible with no other known neurochemical interactions (4, 34). In preclinical studies it was determined

that 65-80% orexin receptor occupancy is required to promote sleep (11). The human plasma suvorexant concentration measured to correspond to 65% OX_2R occupancy is 0.33 μM, free and bound, which is the minimum percentage associated with predicted sleep-promoting efficacy at this receptor (7). Mean suvorexant plasma concentrations fell below this occupancy 8 hours following 10-mg and 20-mg doses which indicates that the sleep-promoting effects of suvorexant should not persist into waking hours at these doses (7). In addition, with a half-life ranging from 9-13 hours, the effects of suvorexant are expected to be maintained throughout the entire sleep period, reducing the number of awakenings associated with insomnia (7).

The recommended dose for suvorexant is 10-mg and it is to be taken once nightly within 30 minutes of going to sleep, but not within 7 hours of the anticipated time of wakening (8, 24). Although the lowest possible dosage should be used, if the 10-mg dose is well-tolerated but not effective, the dosage can be increased to a daily maximum of 20-mg (12, 24). Patients who are prescribed the higher 20-mg dose are advised against next-day activities, such as driving, where alertness may be compromised (8, 24, 39). The median time to maximum plasma concentrations (T_{max}) is 2 hours (range 30 min to 6 hours) under fasting conditions, while administration following a meal high in fat can delay T_{max} by as much as 1.5 hours (12, 24, 25, 32, 34). For a faster onset of sleep, meals before administration should be avoided (25). Steady state equilibrium can be reached within 3 days of daily administration of suvorexant and the mean bioavailability following a 10-mg oral dose is 82% (12, 24, 25, 32). The mean half-life of suvorexant is 12 hours (34). Suvorexant concentrations increase with female sex and obesity, but kinetics are not affected by age or race (8, 25, 34). The drug is 99.5% protein-bound to plasma proteins

and binds to both human serum albumin and α-1-acid glycoprotein (24, 25). The drug does not appear to readily distribute into red blood cells (24, 25). Suvorexant metabolism is the primary route of elimination for the drug, with cytochrome P450 (CYP) 3A being reported as the primary contributor, with minor contribution from CYP 2C19 (25, 32). Suvorexant and its hydroxylated metabolite, having no expected pharmacological activity, are the major species in circulation (25). Patients who are prescribed suvorexant should not take other medications which are associated with CYP450 3A enzyme systems and should avoid grapefruit juice as it could reduce metabolism of the drug (4, 34). Potent CYP 3A inhibitors can cause suvorexant to exceed therapeutic thresholds as plasma concentrations are increased, whereas CYP 3A inducers can decrease suvorexant plasma concentrations (4, 25). Suvorexant itself is a mild CYP 3A inhibitor but is not expected to cause significant inhibition of CYP 1A2, CYP 2B6, CYP 2C8, CYP 2C9, CYP 2C19, or CYP 2D6 (4, 24). If patients are taking moderate CYP 3A4 inhibitors (i.e. diltiazem or ketoconazole), the suvorexant dose should be reduced to 5-mg daily (8, 12, 24, 25). Suvorexant is not recommended in combination with strong CYP 3A inhibitors (24, 25). Suvorexant administration with oral contraceptives or warfarin is not expected to cause inhibition of these compounds (8, 24). Alcohol and suvorexant do appear to have additive effects, particularly on psychomotor performance, and administration of suvorexant with other central nervous system (CNS) depressant drugs is not recommended due to potential additive effects (24). The use of suvorexant with other insomnia medications is also not recommended (24, 25).

Adverse events have been reported in studies following higher doses of suvorexant. Following 40-mg and 80-mg doses, the most common patient-reported adverse events were

abnormal dreams, somnolence, headache, dizziness, upper respiratory tract infection, and urinary tract infection (11, 16). Other events reported have included instances of sleep paralysis and visual hallucinations, as well as unusual nighttime activities and suicidal ideation in doses exceeding 20-mg (8, 15, 16). Side effects may also include dry mouth and fatigue (34). It has been noted that the side effects of suvorexant seem to be dose-related (16). However, there were some initial concerns by the FDA in the development of suvorexant regarding next-day somnolence, inability to decide against driving while still under its effects, sleep walking, vivid dreaming, and other abnormal activities (5). The risk for impacts on driving performance and other activities that require mental alertness can be increased if suvorexant is taken with less than a full night of sleep, at higher than the recommended dosage, or if taken in combination with other CNS depressants (24, 25). Patients who have a history of drug abuse, use suvorexant in combination with other drugs or alcohol, or who use suvorexant for a prolonged period of time are more likely to abuse suvorexant (25). Suvorexant abuse can lead to increased risks of impaired reaction times while driving skills and somnolence (25). However, there is no evidence of physical dependence on suvorexant following prolonged use (25).

Vermereen et al. performed two driving studies following use of suvorexant, one in healthy non-elderly volunteers and one in healthy elderly volunteers (40). The first study examined volunteers aged 23-64 and evaluated next-morning driving performance after single, repeated doses of 20- and 40-mg of suvorexant or placebo over 8 days. Driving performance was assessed using standard deviation of lateral position (SDLP) in a standardized on-the-road driving test for those administered suvorexant versus placebo. Zopiclone was used as a control for days 1 and 8 and placebo was given in between. A

word learning test and body sway were also implemented. Five driving tests were prematurely stopped by four females due to drowsiness. While there were no clinically relevant findings for the study, it was noted that measurable impairment occurred compared to placebo, but less than those observed for alcohol blood concentrations of 0.05 g/dL. The premature stoppage of the study by the individuals who reported excessive drowsiness may indicate that some individuals may experience next-day impairment that hinders driving (40). A similar study was performed in 24 elderly volunteers aged 65-80 years administered 15-30 mg doses of suvorexant or placebo in the same fashion. The results were comparable to the study in healthy younger volunteers, but it was advised that individuals taking suvorexant avoid next-day activities such as driving due to inter-individual variability and the small sample size of the study (41).

The use of suvorexant in combination with alcohol was also studied to determine additive or synergistic effects of co-administration, and to further assess safety and tolerability (23). Pharmacokinetics and psychomotor performance were assessed for healthy subjects who had been administered a 40-mg oral dose of suvorexant with and without alcohol. There were no indications that suvorexant and alcohol had pharmacokinetic interactions, and the most common adverse effects observed after co-administration were dizziness, nausea, somnolence, and headache. Suvorexant administered alone and with alcohol was well-tolerated, but minor decreases in oxygen saturation were observed in subjects given both substances, suggesting that the respiratory depression effects were attributed to alcohol (23). Although pharmacokinetic parameters were not affected by alcohol administration, suvorexant in combination with alcohol had negative effects on psychomotor performance. These effects included additive negative

effects on sustained attention/vigilance, reaction time, working memory, and postural stability, which exceeds those observed with alcohol alone. The conclusion of the study was that suvorexant should not be taken in combination with alcohol, much like other insomnia medications (23).

It has been noted that the effects on insomnia are quite modest at the low doses available, but at the higher doses where greater effects can be seen the adverse effects also increase (34). In addition, the drug can be expensive which can deter its use over other therapeutics (34). With the novelty of the drug, wholesale prices have averaged $315-340 for 30 tablets and is only available in one oral formulation (8). However, as a novel hypnotic, suvorexant is expected to feature in both antemortem and postmortem toxicology investigations.

Detection Methods

Relatively few analytical methods have been published that describe the analysis of suvorexant in biological samples (**Table 1.1**). Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) have been used to detect suvorexant in plasma, urine, blood, and tissues. Of the published reports to date, there is only one study that examines suvorexant in postmortem casework. This highlights the gap in literature and the need for additional research.

Table 1.1. Summary of published analytical methods for the detection of suvorexant.

Matrix	Volume	Extraction	Internal Standard	Instrumentation	Column	Ionization	LOQ (ng/mL)	Reference
Plasma	0.1 ml	LLE	$^{13}C^{2}H_{3}$-suvorexant	LC-MS/MS	C18	APCI	1	(42)
Urine	2 ml	LLE	estazolam-D5	GC-MS	DB5-MS	EI	10	(43)
Urine	1 ml	LLE	estazolam-D5	LC-Q/TOF-MS	EC-C18	ESI+	5	(44)
Plasma	0.2 ml	LLE	rivaroxaban	LC-MS/MS	C18	ESI+	0.33	(45)
Plasma	0.1 ml	LLE	carbamazepine	LC-MS/MS	C18	ESI+	0.16	(46)
Urine	0.25 ml	D-LLE	carbamazepine	LC-MS/MS	C18	ESI+	0.27	(47)
Urine, blood, tissues	0.2 ml/ 0.2 g	PPT/ Captiva ND	diazepam-D5	LC-MS/MS	PFP	ESI+	1	(48)

LLE, liquid-liquid extraction; D-LLE, dispersive LLE; PPT, protein precipitation; Captiva ND, phospholipid removal device.; LC-MS/MS, liquid chromatography tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; LC-Q/TOF-MS, liquid chromatography-quadrupole/time of flight-mass spectrometry; APCI, atmospheric pressure chemical ionization; EI, electron impact; ESI, electrospray ionization.

The first analytical method describing the detection of suvorexant in human plasma was performed by Merck & Co. using liquid chromatography tandem mass spectrometry (LC-MS/MS) (42). The method employed a 96-well liquid-liquid extraction (LLE) using methyl *tert*-butyl ether (MTBE) as the extraction solvent. Separation was achieved using a Waters Atlantis dC18 column (2.1 x 50 mm x 3 µm) with isocratic elution of 30/70 (v/v %) of 10 mM ammonium formate. The isotopically labeled internal standard ($^{13}C^{2}H_{3}$-suvorexant) was manufactured in-house and used for the quantification of suvorexant. Atmospheric pressure chemical ionization (APCI) was used in positive mode with multiple reaction monitoring. The transition monitored for suvorexant was m/z 451 → 186 and the transition for the internal standard was 455 → 190. The assay was validated over a linear range of 1-1,000 ng/mL with a limit of quantitation (LOQ) of 1 ng/mL in human plasma (42). Extraction recovery was only ~50% but the authors note that the use of the stable isotope internal standard compensates for variability across plasma matrices. Accuracy ranged from 96-105% of the expected concentration and inter- and intraassay precision were within 10%. Suvorexant was stable in human plasma held at room temperature for at least 6 hours following thaw, and samples stored at -20°C for a period of 25 months were found to be stable. This method was then applied to human plasma samples that were collected 2 hours following oral administration of the drug at a 10-mg dose to 6 subjects. The method was able to detect suvorexant in all plasma samples within the established range and was reproducible, demonstrating its applicability to clinically relevant doses of suvorexant (42). However, this study has limitations in that it only examined suvorexant plasma concentrations from known dosing of healthy subjects in a clinical setting. In forensic casework, the prescription dosage may differ from the actual administered dosage,

and in postmortem work human plasma is not as commonly analyzed as other biological specimens such as whole blood, urine, or tissues. In addition, while this method is robust, it only employs one precursor to product ion transition which is not generally acceptable for forensic use. A minimum of two ion transitions are required for identification purposes so that ion ratios can be compared (49).

Methods to detect suvorexant in urine were previously developed in our laboratory using GC-MS and liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS). The first GC-MS method by Carson et al. isolated urine via liquid-liquid extraction (LLE) with ether/toluene (50:50) as the organic solvent. At the time of method development, solid phase extraction (SPE) using copolymeric ion exchange columns indicated that suvorexant eluted in the neutral drug fraction (i.e. organic wash). In the absence of a commercially available deuterated analog, estazolam-D5 was used as the internal standard (IS) due to its structural similarity to suvorexant. These similarities included a chlorine moiety, a triazole, and a 7-memebered azapine/azepane ring (43). Method development was initially performed on a traditional DB5-MS column (30 mm x 0.25 mm) with a film thickness of 0.25 µm, but suvorexant eluted at nearly 30 minutes due to its high boiling point (669°C). Development was then continued using a DB-5MS column (30 mm x 0.25 mm) with a reduced film thickness of 0.1 µm, which decreased the elution time to 11.7 minutes. Quantitation of suvorexant and estazolam-D5 was performed on an Agilent 5975C Mass Selective Detector in selected ion monitoring (SIM) mode with electron impact (EI) ionization at 70 eV. The ions monitored for suvorexant were m/z 450.2, 186.1 and 104.1, with 186.1 being the quantitation ion. The ions selected for estazolam-D5 were m/z 299.1, 264.1, and 219.1, with 299.1 being the quantitation ion. The

method was validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology (50). Analytical recovery for the method averaged 106% with LOD and LOQ of 10 ng/mL. The linear range was 10-1,000 ng/mL with accuracy ranging from 98-101%. Intra- and inter-assay precision were within 20% and there were no interferences detected from fifty common drugs or matrix. Carryover following injection of 1,000 ng/mL standard was mitigated with the use of 12 pre- and post-injection methanol needle washes. Processed stability was evaluated, and it was found that the internal standard was not stable past 48 hours on the room temperature autosampler, lending that samples stored longer than 24 hours at this temperature should not be analyzed. This method highlighted the limitations with GC-MS analysis for suvorexant, in that retention times using traditional screening parameters and columns could cause the drug to go undetected in a typical acquisition window. Mitigation of these problems include the reduction of the column thickness and the use of a SIM method to increase analytical sensitivity (43).

A second urine method validated in accordance with SWGTOX recommendations for forensic use was developed by Sullinger et al. with LC-Q/TOF-MS (44). Like the method by Carson et al, this method employed estazolam-D5 as the IS in the absence of a stable isotope suvorexant standard and used LLE with ether/toluene (50:50) (43, 44). Analytes were separate on an Agilent 1290 Infinity binary LC system with a Poroshell 120 EC-C18 column and EC-C18 guard column. Gradient elution of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was performed to achieve the desired chromatographic profile, and detection of suvorexant and IS ions was achieved with an Agilent 6530 Accurate-Mass Q/TOF operated in positive electrospray ionization (ESI)

mode. The product ion transitions monitored for suvorexant were m/z 451.1644 → 186.0664 (quantifier ion) and 451.1644→ 104.0493 (qualifier ion) at a collision energy of 50 eV. The transitions monitored for estazolam-D5 were m/z 300.1059→ 272.0875 (quantifier) and 300.1059→ 210.1076 (qualifier) at a collision energy of 30 eV. The method was validation over a range of 5-250 ng/mL in urine using a quadratic weighted (1/x) calibration model. The LOD and LOQ were 0.5 ng/mL and 5 ng/mL, respectively, with intra- and inter-assay precision <8%. The bias ranged from -2 to 4% and no carryover was detected. No interferences were reported from the internal standard, matrix, or other common drugs (50 analytes evaluated). This method provided an easily adapted LLE that labs can apply to their existing protocols using newer LC-Q/TOF-MS technology for toxicological analysis (44).

An increasing number of methods for suvorexant are being developed for use with LC-MS techniques. The analysis of suvorexant in urine has also been described by Iqbal et al. using a unique dispersive liquid-liquid micro-extraction and LC-MS/MS (47). Suvorexant was isolated from urine using a simplified dispersive liquid-liquid micro-extraction followed by ultrasound assisted back extraction of solidified organic droplets (DLLME-SFO-UABE), with acetonitrile and 1-undecanol as the dispersive and extraction solvents, respectively. LC-MS/MS was used in positive electrospray ionization mode and using multiple reaction monitoring. The suvorexant transitions used were m/z 451.12 → 104.01 and m/z 451.12 → 186.04. The internal standard, carbamazepine, had one transition of m/z 237.06 → 194.1. Separation was achieved using a Waters Acquity UPLCCSH™ C18 column and isocratic elution of 15 mM ammonium acetate: acetonitrile: formic acid (15:85:0.1%; v/v/v). This method too was validated according to SWGTOX

recommendations over a range of 0.27-1000 ng/mL in urine using a linear weighted (1/x2) calibration model (50). The LOD was 0.1 ng/mL and the LOQ was 0.27 ng/mL with precision and bias within acceptable ranges. The method was free from carryover and ion suppression was negligible. Stability of suvorexant in urine was described at different storage temperatures and was found to be stable up to 24 hours at room temperature, for up to one month at -80°C, and for 24 hours in the autosampler in which the temperature was not specified. Suvorexant was stable after three freeze-thaw cycles according to the authors (47). While this method demonstrated improved sensitivity over the previous methods for urine, the use of an internal standard that is not isotopically labeled could pose problems during analysis of samples in which carbamazepine is also present, as it is also a commonly encountered drug in forensic toxicology. In addition, SPE, LLE, and protein precipitation (PPT) are commonly used in forensic laboratories, but the use of DLLME-SFO-UABE might be limited.

This research group has also published methods for the detection of suvorexant in plasma using LC-MS/MS (45, 46). In one method, suvorexant was isolated from rat plasma using LLE with diethyl ether as the organic extraction solvent. The internal standard used was rivaroxaban, and detection was performed using LC-MS/MS in positive ESI mode with multiple reaction monitoring. The transitions for suvorexant were m/z 451.12 → 104.01 and m/z 451.12 → 186.04 and the transition for rivaroxaban was m/z 436.10 → 144.93. Separation was achieved on a Waters Acquity BEH™ C18 column with an isocratic elution consisting of acetonitrile and 15 mM ammonium acetate (85:15, v/v%). The method was validated with SWGTOX guidelines and provided a linear ($1/X^2$) concentration range of 0.33-200 ng/mL in rat plasma (50). The LOD was 0.1 ng/mL and

the method met all other standards regarding precision, bias, carryover, matrix effects, and interferences. This study also included dilution integrity experiments in which suvorexant met acceptability criteria following dilution (2x and 4x) of positive samples with blank plasma (45). The authors describe a separate assay for the detection of suvorexant in fortified human plasma (46). Suvorexant and the internal standard, carbamazepine, were isolated using LLE with MTBE as the extraction solvent. The Waters Acquity BEH™ C18 column was used with isocratic elution of 10 mM ammonium acetate/acetonitrile/formic acid (15:85:0.1%; v/v/v) for the separation of suvorexant and carbamazepine. LC-MS/MS was used in positive ESI mode, and the transitions for suvorexant and carbamazepine were the same as in the previous report for urine (47). Using a weighted ($1/X^2$) linear model, the calibration range was 0.16-250 ng/mL in plasma with an LOQ of 0.08 ng/mL. The method met acceptability for bias, precision, carryover, matrix effects, and interference from 11 other drugs (46). While these methods are sensitive and selective for the detection of suvorexant in plasma, this matrix is not commonly used in postmortem forensic casework unless comparing antemortem and postmortem concentrations, and specimens such as blood or tissues could be more informative in a death investigation.

There has only been one analytical method for the detection of suvorexant in urine, blood, and tissues that has been used on authentic case specimens in the literature to date. In 2018, Waters et al. described the detection of suvorexant in three forensic autopsy cases using GC-MS/MS and LC-MS/MS, providing the first report of suvorexant distribution in the body following death (48). In this study, samples were first screened for suvorexant by GC-MS/MS. Like in previous studies, the authors acknowledge that using traditional GC-MS conditions resulted in the elution of suvorexant at 23.7 minutes which could cause it

to go undetected during data acquisition (43, 48). The late elution of suvorexant, due to its high boiling point, was mitigated in this study using tandem GC columns. With tandem columns, the total run time was reduced to 9.16 minutes with a suvorexant retention time of 5.25 minutes. Following positive identification with GC-MS/MS, fluids and tissues were prepared for LC-MS/MS quantitation using diazepam-D5 as the internal standard. Urine and blood samples were subjected to protein precipitation with acetonitrile. Tissues were prepared by homogenization and protein precipitation of the resulting fluid. Supernatants of all precipitated samples were subsequently subjected to Agilent Captiva ND Lipids cartridges for clean-up. Separation of analytes was achieved using a Hypersil GOLD PFP column and gradient elution of 0.1% formic acid in water (A) and 0.2% formic acid in acetonitrile (B). The LC-MS/MS source was operated in positive ESI mode with suvorexant transitions of m/z 451 → 186 and m/z 451 → 104 being used, as well as diazepam-D5 transition m/z 290 → 198. The method was validated according to SWGTOX and used to analyze postmortem case specimens. The LOD and LOQ for both blood and urine were 0.5 ng/mL and 1 ng/mL, respectively. The calibration range was from 0.5-500 ng/mL using a linear model. Precision, bias, and matrix effects fell within acceptable ranges, and there was no carryover or interferences from 20 common analytes observed. Stability of QC samples following storage at 4°C for 6-24 hours demonstrated 92-99% accuracy (48).

Suvorexant in Toxicology Casework

Suvorexant casework has not been adequately described in the literature and few reports have been made available to indicate its prevalence in forensic cases. Therefore, little is known of its role in human performance toxicology or in forensic investigation. No

studies are available concerning antemortem suvorexant concentrations outside of the clinical setting, and the only study to date detailing the analysis of suvorexant in postmortem specimens was published in 2018 by Waters et al. (48). The method detected suvorexant in three forensic autopsy cases using LC-MS/MS.

The first of the three cases involved a female in her forties that was found deceased on the beach. It was determined the cause of death was drowning and she had been prescribed depression medications along with suvorexant for the treatment of insomnia. Other drugs found during toxicology were 7-aminoclonazepam, flunitrazepam, olanzapine, and quetiapine. Suvorexant was present in right heart blood (455 ng/mL), left heart blood (491 ng/mL), left femoral blood (421 ng/mL), liver (201 ng/mL), kidney (280 ng/mL), spleen (36 ng/mL), pancreas (55 ng/mL), lung (122 ng/mL), muscle (93 ng/mL), and fat (359 ng/mL). Police records had indicated that the decedent had been prescribed the 20-mg dose of suvorexant to take once nightly for insomnia. Previous clinical studies on suvorexant administered to 5 healthy men in a 50-mg oral dose indicated average peak plasma concentrations of 392 ng/mL (30). Blood concentrations, which accounted for most of the suvorexant detected in this case, were well above expected peak plasma concentrations.

The second case involved a male decedent found lying prone in a bedroom with no remarkable injuries. At autopsy a bluish-green substance was found in the oral cavity, esophagus, and stomach contents indicating that the substance Rohypnol® may have consumed. The man had been prescribed Rohypnol® as well as a 20-mg nightly dose of suvorexant. Other significant findings were the presence of 15 additional drugs at the time of autopsy, with elevated levels of promethazine, nifedipine, chlorpromazine, and zotepine

present. Suvorexant was detected in right heart blood (15 ng/mL), left heart blood (17 ng/mL), left femoral blood (11 ng/mL), urine (<1 ng/mL), liver (6 ng/mL), kidney (4 ng/mL), spleen (<1 ng/mL), pancreas (1 ng/mL), lung (40 ng/mL), muscle (<1 ng/mL), and fat (5 ng/mL). The concentrations of suvorexant were less than those reported in clinical findings for plasma. Due to the presence of several drugs, some at toxic levels, the cause of death was determined to be combined drug intoxication.

The third case was another male decedent in his 80s who had reportedly become blind due to diabetes 10 years prior. The man was reported to be found hanging from the neck, and autopsy showed furrows in the neck with subcutaneous bleeding, and a fractured thyroid cartilage. The manner and cause of death were suicide by hanging. In addition to diabetes, the man suffered from anxiety and was prescribed 20-mg suvorexant tablets, triazolam, and other medications that were not described. Ethanol was found in the blood at a concentration of 0.02 mg/mL and other drugs found in the cardiac blood were bisoprolol, N-desalkylflurazepam, and triazolam. Suvorexant was found in right heart blood (138 ng/mL), left femoral blood (155 ng/mL), urine (10 ng/mL), liver (158 ng/mL), kidney (232 ng/mL), spleen (31 ng/mL), pancreas (24 ng/mL), lung (41 ng/mL), muscle (30 ng/mL), and fat (278 ng/mL) (48).

The authors acknowledge that the distribution of suvorexant varied significantly between the three cases. While the first case had the highest suvorexant concentration in blood, the second and third cases exhibited the highest concentrations in lung and fat respectively. Postmortem redistribution (PMR) could not be identified due to the lack of a significant pattern but is often used to help interpret postmortem concentrations of a drug. In the three cases, the central/peripheral (C/P) blood ratios ranged from 0.89-1.36 which

suggests that suvorexant did not have an obvious tendency to redistribute into central blood during the postmortem interval (48). Although this is the first case report to be published detailing the analysis of parent drug in forensic specimens, no studies have been published to date detailing the analysis of suvorexant metabolites in forensic casework.

Metabolism

In vitro Approaches to Metabolism

Understanding the metabolism of a therapeutic or drug is essential, so that adverse drug reactions may be predicted during the preclinical stages of its development. Preclinical development of compounds usually involves investigation of pharmacological properties and heavily examines metabolism of these compounds. When drugs are metabolized in the body, production of active metabolites can potentially cause toxic effects, so a thorough evaluation of biotransformation is necessary using *in vivo* and *in vitro* methodologies in animal models (51). Metabolic characteristics that are usually studied include the drug's metabolic stability, metabolic route, which enzyme systems are responsible for drug metabolism, and how these systems are inhibited for identifying potential drug-drug interactions (51).

The two stages of metabolism involve phase I, where nonpolar species are converted to a polar species, which can subsequently be conjugated with a glucuronic acid, sulfate, glycine, methyl, or acetyl group in phase II metabolism. These biotransformations primarily take place in the liver which also houses many cytochrome P450 (CYP) enzymes that are responsible for various metabolic reactions. The CYPs primarily responsible for drug metabolism are CYP 3A4, 2C19, 2D6, 2C9, and 1A2, while others exist that contribute to metabolism to a lesser extent (51, 52). CYP 3A4, which is present in the liver

in high concentrations, is one of the most important isoforms involved in human drug metabolism. It is involved in the metabolism of the majority of drugs covering a wide range of uses, which is problematic from the standpoint of drug-drug interactions and interactions arising from an individual's diet which can also affect drug interactions (i.e. grapefruit juice) (52). However, it is common for a drug to be subjected to more than one metabolic pathway, and identifying these pathways is important if adverse interactions are to be avoided.

Many *in vitro* experimental approaches have been developed for estimating *in vivo* human drug metabolism by using systems derived from human liver. These have included microsomes, supersomes, S9 fractions, and human hepatocytes, among others (51). Each of these systems can help identify major metabolic pathways for a compound, but all have their own advantages and vary in the information they provide. Human liver microsomes (HLMs) are useful in identifying metabolites and predicting drug clearance and are the most popular *in vitro* model in part due to being one of the most characterized models (51). HLM reactions are typically used first to evaluate metabolism formation rates using enzyme kinetic analysis. HLMs contain many drug-metabolizing species such as cytochrome P450s (CYPs), flavin monooxygenases, and UDP glucuronyl transferases which require NADPH regenerating systems, exogenous cofactors, to measure oxidase activity in producing phase I metabolites. Phase II metabolites can also be studied by the addition of uridine-5'-diphospho-α-D-glucuronic acid (UDPGA) cofactors. Metabolite identification is typically performed by incubation with HLMs followed by high resolution mass spectrometry (HRMS) techniques which allow for structural elucidation with increased mass accuracy. While useful for predicting likely metabolism, HLMs are

disadvantageous in that their high concentration of CYPs makes it difficult to translate quantitative values to in vivo human metabolism where enzymes are present in lesser amounts (51).

To complement HLMs, supersomes are often used to investigate biotransformations *in vitro*. This approach uses insect cells that would usually be devoid of endogenous CYP or uridine 5'-diphospho-glucuronsyltransferase (UGT) activity but contain human CYP- or UGT-expressing hepatocyte endoplasmic reticulum vesicles and baculo virus. The advantage of using supersomes, or bactosomes, is that single CYP isoform contribution can be identified which is important for contraindication of drugs by their expected drug-drug interactions as well as influence of polymorphisms on metabolism (51). Like studies with HLMs, addition of cofactors such as NADPH-regenerating systems are needed for activity (51). Recombinant enzyme systems such as these have the added advantage of ease of preparation versus HLMs which are more difficult to obtain (53). S9 fractions are another method that has been used for describing metabolic systems, although not as commonly used as human liver microsomes or supersomes. S9 fractions consist of microsomal and cytosolic fractions from the liver and require NADPH for activity of CYP enzymes. Advantages of using this method are that both phase I and phase II metabolites can be generated, but the lower activity of these fractions compared to the two previously described methods can allow for some metabolites to go undetected (51). Another popular way to study phase I and phase II metabolism *in vitro* is using human hepatocytes which have good correlations for *in vitro-in vivo* studies. Human hepatocytes have been used to study metabolic stability, drug-drug interaction potentials, and metabolic profiling. These hepatocytes are generally reflective of heterogeneity of human liver expression of CYPs

and when cryopreserved, enzyme activity can be retained for phase I and phase II reactions (51).

While useful for the purposes of predicting likely metabolism, there are some intrinsic differences that can arise between *in vitro* and *in vivo* data due to the nature of these modeling experiments. One reason may be that metabolism can take place in areas of the body other than the liver, while most of these approaches focus on liver-derived mechanisms, so metabolism can be underestimated. For instance, orally administered drugs will have a higher concentration in the gut, which is rich in CYP 3A4, particularly in the small intestine. High-clearance drugs are more susceptible to these types of underestimations. In addition, high concentrations of drug used in in vitro metabolic studies that exceed the anticipated therapeutic range of the compound can influence its metabolic behavior due to saturation of the enzymatic system (53). One of the biggest disadvantages of most of the *in vitro* methods discussed is the discrepancies arising from inter-individual variability, or genetic polymorphisms, in enzymatic activity. An example of an enzyme system with a high degree of polymorphism is that of CYP 2D6 in which the three phenotypes are slow-metabolizers, rapid-metabolizers, and ultrarapid-metabolizers, which can affect toxicity of drugs on individuals. CYP 2C19 is also known for its polymorphism. Most CYP isoforms are known to have some degree of genetic polymorphism associated with them, but inter-individual variability may also be affected by enzyme induction or inhibition, diet, age, chronic smoking, and overall health of the individual (52). This can be overcome in part by using pooled HLMs and hepatocytes, but is still an important consideration when conducting *in vitro* assays (51-53).

Currently, HRMS and nuclear magnetic resonance (NMR) assays are the most powerful tools in metabolomic studies, as they can identify individual metabolite profiles using both *in vivo* and *in vitro* approaches. Methods that use mass spectrometry are becoming increasingly used over NMR techniques due to their high sensitivity and wider availability of instruments. While they do not have the precise structural identification capabilities of NMR, HRMS techniques can be used to perform structural elucidation by using accurate mass and MS/MS fragmentation patterns (54, 55). To help with the identification of individual metabolites *in vivo*, radio-tracing or radiolabeling has been utilized. In this approach, subjects are administered a radiolabeled dose of the compound and elimination is monitored based on the radioactive dose recovered and analytes can be deduced in this manner. This requires synthesis of a radiolabeled compound which can often be expensive and requires a facility capable of housing radioactive compounds (54). Conversely, drug metabolites can be monitored using treatment and placebo groups, and identifying metabolites based on mass spectral differences between the treatment group (which will contain metabolites) and the control group which should contain no metabolite. Differences can be assessed by peak recognition, ion identification, and analysis of ion abundance, either performed manually or by a data matrix which can incorporate centroid profiles, isotope patterns, and mass filtering for very large data sets (54, 56). It is due to these capabilities that LC-MS techniques are becoming more widely used in metabolite identification and description during metabolomic studies.

Suvorexant Metabolism

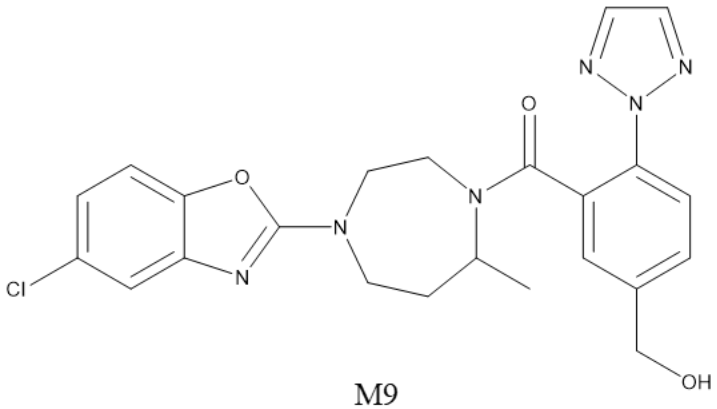
The metabolism of suvorexant *in vivo* and *in vitro* has only been described in one report. In this report, the metabolism and elimination of suvorexant was characterized in

healthy subjects that were administered [^{14}C]-suvorexant and unlabeled suvorexant. Metabolism of suvorexant was also investigated using a number of *in vitro* techniques to identify enzymes responsible in the biotransformation of the drug (22).

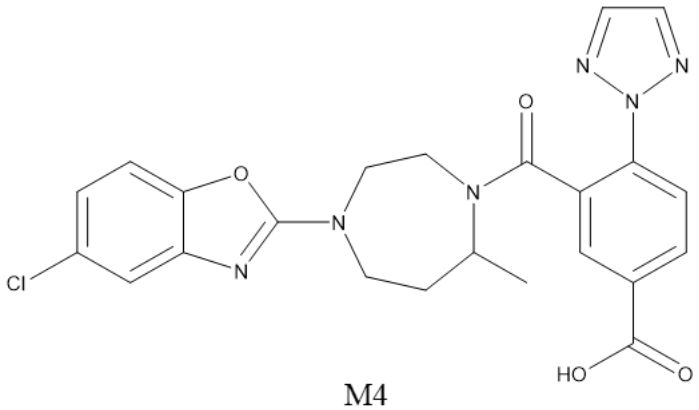
A clinical study with the administration of radiolabeled suvorexant was performed on males 18-45 years of age who were healthy and non-smoking. A single 50-mg oral dose of [^{14}C]-suvorexant was administered to subjects following an overnight fast. Over a 28-day period, samples of urine, blood, and feces were collected. Plasma samples were also collected pre-dose and up to a period of 504 hours following dosing. Radioactivity of the plasma samples was performed using liquid scintillation counting (LSC) and suvorexant concentrations were determined using LC-MS/MS over a range of 1-1000 ng/mL. Following the 50-mg dose, study of human plasma showed that followed the radioactive dose, suvorexant had a mean C_{max} of 1.6 µM (720.32 ng/mL), a T_{max} of 1.5 h, and a terminal $t_{1/2}$ of 12.3 h. Results indicated that 12-35% of plasma radioactivity was contributed from unchanged suvorexant. Excretion of radiolabeled suvorexant was determined to be primarily fecal elimination (66%) followed by urine (23%) (22).

Metabolism was assessed by overall recovery of [^{14}C]-suvorexant in urine, feces, and plasma measured by LSC. Metabolite profiles were obtained using HRMS with offline radiometric identification and quantitative analysis was performed based on the % radioactivity compared to the initial dose. Proposed metabolites were based on structural changes to the key fragment ions used in suvorexant identification (m/z 451.1644 and m/z 186.0662). Metabolites M4, M9, and M10a were identified and compared to standards (manufactured by Merck & Co.) for confirmation (**Figure 1.3**). Of the other metabolites identified, structural elucidation and confirmation was performed for four species (M7a,

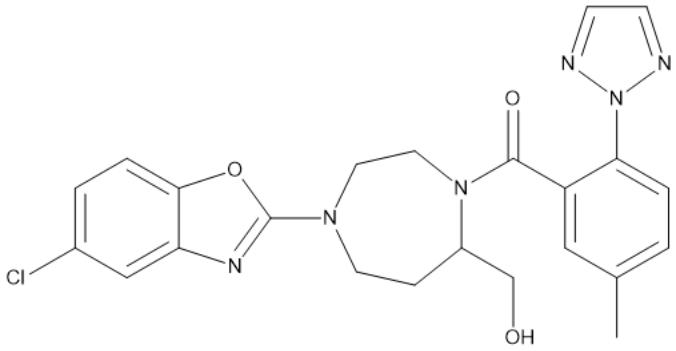
M8, M16, and M17) using high resolution solution NMR analysis. Metabolites were confirmed using both NMR and MS/MS after production using Codexis® BM3 Cytochrome P450 variants. Results showed that these four metabolites are characterized by hydroxylation of the chlorobenzoxazole, with M7 and M17 having an additional hydroxylation on the phenyltriazole (**Figure 1.4**) (22).



M9



M4



M10a

Figure 1.3. Chemical structures of notable hydroxylated metabolites of suvorexant (M9, M4, and M10a) that were compared to standards.

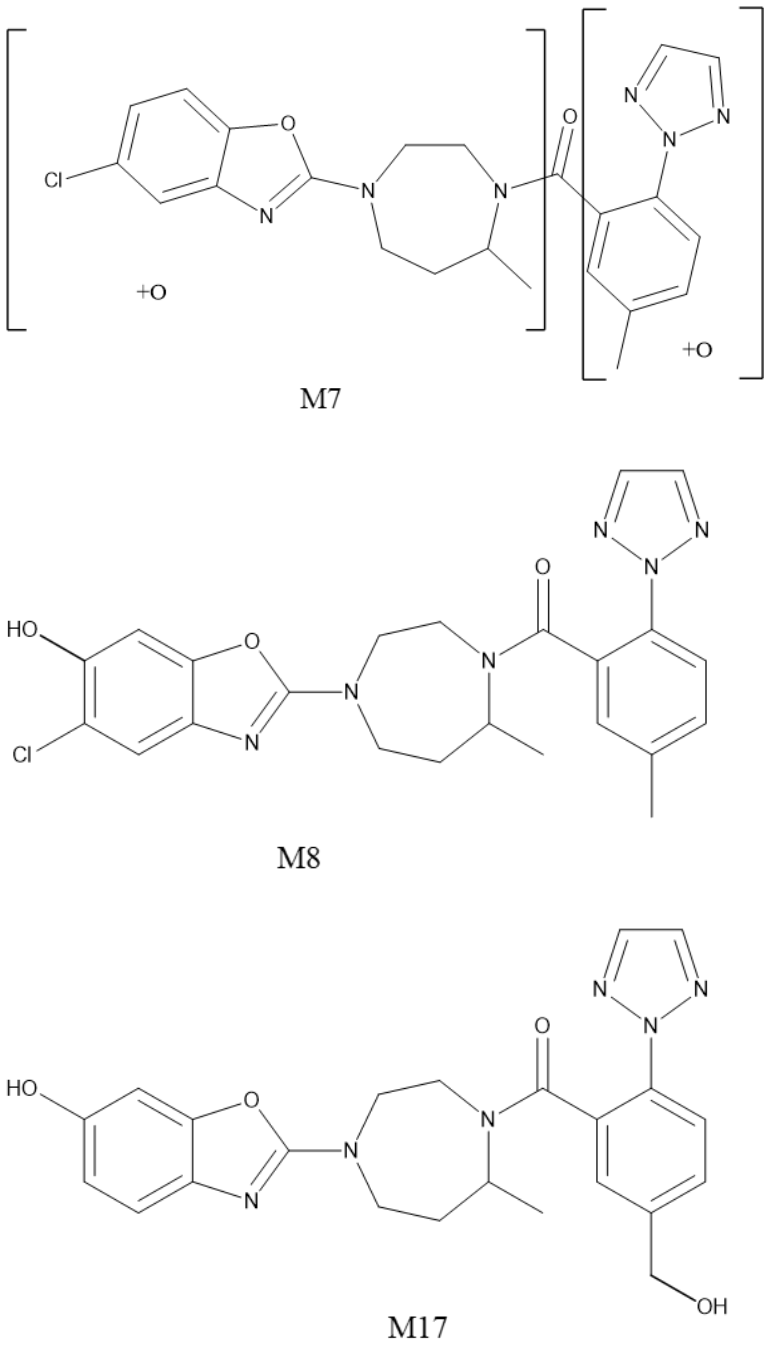


Figure 1.4. Chemical structures of additional hydroxylated metabolites of suvorexant (M7, M8, and M17) identified by HRMS structural elucidation and NMR.

The carboxylated metabolite of suvorexant, M4, and the corresponding glucuronide of that metabolite, M19, were found to the be the primary urinary metabolites (4.1% and

5.3% of the total dose, respectively). The glucuronidated metabolites M3, M12, and M11 were detected in urine at 3.8%, 2.7%, and 1% radioactivity of the original dose. No radioactively labeled parent suvorexant was detected in urine. For feces, the primary species was M4 (18%) followed by M18 (10.6%). The hydroxylated M9 metabolite, and the M10a metabolite produced from hydroxylation of the methyldiazepan, were also present in feces at 9% each. Other minor metabolites were detected in feces to include M6b/c, 7a, and 13a/b/c, but minimal parent drug was present. The plasma concentrations measured reflected the highest amount of suvorexant (30.1%) and its hydroxylated metabolite (36.5%). Plasma also contained M12 (glucuronidated M10a) at 12.2% and showed minor contributions from metabolites M4, M7a, M8, M10a, and M17 (22).

Following identification of potential metabolites, plasma samples from a multiple rising dose study were analyzed for the presence of unlabeled suvorexant and its metabolites. Forty healthy males aged 18-45 participated in a controlled, double-blind, randomized multiple dose study in which subjects were either given a suvorexant oral dose of 10, 20, 40, 80, or 100 mg, or were administered a placebo. Plasma was collected pre-dose and up to 72 hours post-dose. Subjects were allowed a 120-day washout period before being administered a matching dose (or placebo) for a 14-day period. Plasma samples were collected pre-dose and up to 96-hours post-dose. Plasma samples were analyzed by HRMS. It was found that results from this study were in good agreement with those from the radiolabeled suvorexant administration study. In addition to the parent drugs and M9 being the most predominant species present, it was observed that M17 was more prevalent in this study and accounted for 17% of the total dose. Ciu et al. also describe the plasma protein

binding of suvorexant metabolites M9 and M17, which are extensively bound to plasma proteins as with the parent drug (22).

To identify the specific enzymes associated with the metabolism of suvorexant, the authors performed cytochrome P450 reaction phenotyping and enzyme kinetics studies. Immuno-inhibition studies using human liver microsomes (HLMs) and monoclonal antibodies (anti-CYP 1A2, 3A4/5, 2C8/18/19, and 2D6). Concurrent monitoring of metabolite formation using HRMS and online radiometric detection using a radiochemical flow detector was performed using a column splitter. In the presence of anti-CYP 3A4/5, the formation of the M8 metabolite was inhibited by 82-100% while M9 formation was inhibited 65-80% depending on the suvorexant concentration (2 or 20 µM). Anti-CYP 2C resulted in 10-30% metabolite formation inhibition at 20 µM of suvorexant, and anti-CYP 1A2 and 2D6 had minimal inhibition of metabolite formation. As a result, CYP 3A is the main enzyme identified in the oxidative metabolism of suvorexant. Specific substrate turnover study with rCYPs only implicated rCYPs 3A4 and 2C19 in kinetic activity (22).

Suvorexant induction and inhibition was studied in a reversible inhibition study using CYPs (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) and HLM activity for those CYPs. Data showed that there was some suvorexant inhibition activity for CYP 2C19 and 3A4, with weak inhibition of the remaining enzymes studied. Inhibition of CYP 3A4 activity in HLMs by suvorexant was determined to be time dependent. Hepatocyte induction was studied using human hepatocytes across a range of suvorexant concentrations (0.1 µM and 20 µM), and mRNA levels and enzyme activity of CYP 3A4, 1A2, and 2B6 were assessed. Overall, CYP 3A4, 2B6, and 1A2 mRNA increases were observed incubations at all

suvorexant concentrations. Decreasing enzyme activity with increasing suvorexant concentrations could be explained by time-dependent inhibition (22).

In summary, the report demonstrated that suvorexant's oxidative metabolites and glucuronidated analogs were the major components found in human urine and feces, while unchanged suvorexant and its hydroxylated M9 metabolite were predominant in human plasma. Data shows that metabolites account for a large portion of the circulating material following oral dosing of suvorexant, and it seems to be well absorbed (>90%) and extensively metabolized. The metabolites described in vitro and in vivo are not expected to contribute to pharmacological activity in humans and the pharmacological effects observed are due to the parent compound alone. Moreover, suvorexant shows low systemic clearance and low first-pass metabolism. The elimination of suvorexant is primarily facilitated by CYP 3A-mediated metabolism and at recommended doses it has low potential for inhibition or induction of major CYPs (22).

Postmortem Redistribution

Postmortem redistribution (PMR) is a significant concern in forensic toxicology casework. Postmortem redistribution is known as the movement of drugs in tissues, organs, and fluids following death (57). Changes that occur after death can cause increases or decreases in drug concentrations which can affect interpretation of drug concentrations (58). Not only are these changes highly variable, but they are also time and site dependent which requires that special attention to where a specimen was sampled from following death (58, 59). In addition, the postmortem stability of a compound is highly dependent on its physicochemical properties. As such, it is difficult to estimate the antemortem concentration for a drug, particularly when postmortem redistribution is significant.

Physicochemical properties of drugs that can greatly affect postmortem redistribution are its size and charge, pKa, apparent volume of distribution, protein binding, partition coefficients, and residual enzyme activity. Environmental factors may include temperature, pH, blood movement between body cavities, repositioning of the body, and bacterial activity (57, 58). Site dependence is an important consideration for collection and interpretation of samples, as drug distribution can differ between sites (i.e. higher drug levels in central blood over peripheral blood). The difference in concentration between central and peripheral sites is known as the central/peripheral (C/P) ratio. Peripheral blood is much less susceptible to the effects of postmortem redistribution and is a more reliable forensic specimen. C/P ratios can be informative about the distribution of drug at the time of death, however they can change depending on the time between death and postmortem examination (57, 60). Comparison of antemortem and postmortem concentrations can provide information on the redistribution of drugs, however antemortem concentrations are often not available for comparison.

Other changes that occur after death can occur due to decompositional changes which can affect drug concentrations. After death, blood and plasma do not easily separate due to hemolysis, so interpretation of blood plasma ratios is usually not possible. This causes difficulties from the standpoint of interpreting clinical therapeutic concentrations which are generally reported for plasma, and most drugs have differential binding to blood and plasma fractions (58, 61). After death, the body becomes increasingly more acidic which can cause drugs to redistribute with the increased permeability of membranes (58, 61). Drugs with high volumes of distribution (>3 L/kg), weak basicity, and lipid solubility are more likely to redistribute after death (58, 59, 62). While the volume of distribution is

known for suvorexant (0.5-0.9 L/kg) and the pKa is reported to be 1.5, there is limited literature regarding its PMR and lipophilicity (63). In addition, only the report by Waters et al. described suvorexant in postmortem specimens in a series of just three cases (48). There was no apparent pattern of PMR in this study but understanding additional physicochemical properties of suvorexant can help predict its behavior before and after death.

Lipophilicity

The lipophilicity of a compound is often used in preclinical drug discovery or development to estimate its likely absorption, distribution, and elimination within the body. Membrane permeability of drugs is highly dependent on its solubility and lipophilicity to reach receptor targets. During drug development a drug must be able to be absorbed and distributed, which is influenced by its ability to undergo passive diffusion (64). Compounds must be sufficiently lipophilic to traverse membranes, but not so lipophilic that they become trapped within the membrane. Partition and distribution coefficients (P and D, respectively) are often used to measure this property, typically expressed as a logarithm (Log P, Log D). Log P refers to the distribution of the unionized compound while Log D is the descriptor for ionizable compounds in a two-phase system (65). When lipophilicity is expressed in this manner it can be the most informative in the application to the absorption, distribution, metabolism, and excretion (ADME) profile of a compound (64). Log P values have been both theoretically and experimentally evaluated for a number of compounds.

To measure Log P experimentally, the most common approach is determining its partitioning between two immiscible phases, most frequently octanol and water. This is

typically performed using the classical shake-flask method in which a known amount of analyte is mixed between these two immiscible phases and then partitioning, expressed as Log P, is the resulting ratio of species present in each phase (octanol/water) following thorough equilibration. This method has traditionally been the most accurate for determining Log P values ranging from -3 to 4, but it does have its disadvantages. These methods may require large solvent volumes, sufficient analyte concentrations for determination of highly lipophilic compounds in the aqueous phase (parts per billion range), and inaccuracies that can be suffered from limited solubility of compounds and adsorption to glass walls (64, 66). For highly lipophilic compounds it is also important to consider that these compounds tend to also have low aqueous solubility which can also result in inaccurate determination of partitioning. In such cases, a co-solvent such as DMSO can be used to increase the solubility of the standard solution. Alternatively, the sample can be dissolved in octanol, and then the amount of drug in the aqueous phase is increased by directly equilibrating with aqueous phase without dilution. After offline separation of phases, common analytical techniques can be used to measure the concentration of analyte in organic and aqueous phases. Analysis time can be reduced by only determining the concentration of analyte in one phase and obtaining the concentration of the other phase by difference from the starting concentration- considering no absorption of analyte to glass occurred (64).

More recently, a popular way to measure partition coefficients is by use of online LC separation procedures, which provide greater speed and simplicity over traditional methods (66). UV spectroscopy and LC can be used to measure concentration of analyte in both organic and aqueous phases. One advantage of LC is the reduction in analyte

concentration required for accurate determination and the elimination of impurities by chromatographic separation (64, 67). Andres et al. (2015) described a method in which equilibration between the phases and analysis of the sample were performed in sample chromatographic vials, but went on to describe the difficulty of LC measurement of concentration in both organic and aqueous phases due to the high viscosity of octanol which can dirty the analytical column. In addition, the low volatility of octanol can prevent it from being used as a solvent in mass spectrometry (MS) detection (64). Another proposed method involves measuring an aqueous standard solution prior to equilibration with octanol, then measuring the aqueous phase again following equilibration. Partition coefficients can be calculated by comparing the peak areas of the standard solution and the aqueous phase. Other experimental approaches to determining lipophilicity have been thin-layer chromatography, electroseparation techniques, and electroanalytical methods (68, 69).

Log P values can be determined theoretically which can help estimate lipophilicity of compounds that would otherwise be difficult to measure and when experimental techniques are not available (68, 70). Computational techniques have been developed utilizing a variety of algorithms. Computational programs have included IAlogP, ClogP, CSLogP, LogPKowwin, xlogP, MILOGP, Hyperchem 7.0, ALogPS, and ACD Labs/Log P (71, 72). Additive atomic contribution methods that take into account "correction factors" such as intermolecular interactions are achieved with ClogP, xlogP, Log Kowwin, MILOGP, HyperChem 7.0, ACD Labs/Log P. Programs that use electrotopological characteristics and E-state indices to predict Log P include ALogPS, IALogP, and CSLogP (71, 72). Methods that use additive constitutive fragment determinations, such as ACD

Labs/Log P and Log KowWin, operate by summing all of the atoms and fragments of a molecule with their relative contributions, and then adjusting the overall hydrophobicity by incorporating a correction factor. The correction factor serves to take into account other interactions that occur within the molecule that can affect lipophilicity. For Log KowWin, the correct factor examines steric interactions between hydroxy and carbonyl substituent groups as well as the linear equation constant (71, 72). For ACD Labs/Log P, follows a similar process and also examines possible tautomerization of compounds and contributions from carbons not belonging to a functional group based on hybridization state (73, 74). On the other hands, programs that use electrotopological characteristics may give better predictions for partitioning of compounds. ALogPS is one of these programs which predicts Log P values by considering both electronic and topological characteristics of molecules, and subsequently assigning E-state values to atoms based on those characteristics and its neighboring atoms (72).

Ultimately, lipophilicity can affect partitioning into tissues and organs and can be an additional predictor for postmortem redistribution. The lipophilicity of suvorexant is unknown and experimental determinations for its partition coefficients have not been made, further highlighting the gap in knowledge that needs to be filled regarding its physicochemical properties.

Matrix Effects & Ion Suppression

Matrix effects are an important consideration when used LC-based techniques. High performance liquid chromatography (HPLC) techniques have been paired with several modes of ionization such as electrospray (ESI), atmospheric pressure chemical (APCI), and atmospheric pressure photoionization (APPI). HPLC-based methods are

becoming the preferred technique for analyzing pharmaceuticals and have applications across a wide variety of disciplines, including forensic toxicology. Mass selective detection in combination with hydrophobic separation make LC-MS techniques powerful analytical tools, and ESI-based LC methods have become the benchmark for identifying drugs and their metabolites in biological specimens (75). It is believed that the high selectivity of LC-MS/MS in combination with successive mass filtration reduce the elimination of co-extractive and co-eluting interferences, which in turn has led to analysis that features minimal specimen preparation and shorter chromatographic retention (75-77). However, it is become well known that LC-based methods are susceptible to interferences, such as matrix effects, that can lead to ion suppression and reduced ionization for target analytes. Analytical data in the forensic toxicology setting must be correctly interpreted and free from these interferences to ensure underestimations and false interpretations are not made (78).

Matrix effects occur when endogenous species altering the ionization efficiency. The increase in efficiency of ionization is referred to as ion enhancement, while the decrease in ionization efficiency is known as ion suppression (75, 78, 79). The successful development of an analytical method is dependent upon a thorough understanding of matrix effects, and many methods fail to adequately address the problem of matrix effects (75). This is an analytical challenge, as matrix effects can affect accuracy and precision of LC-ESI-MS/MS methods and ion suppression can adversely impact the sensitivity of a method (i.e. LOQ) (75, 80). Retention time shifts and changes in baseline response can also occur, as well as imprecise calibration (81, 82). Retention time shifting in LC-MS/MS techniques can be detrimental in methods that employ automation when identifying

compounds such as metabolites whose identification is highly dependent on retention time matching and accurate mass. This can impact the drug development and pharmacokinetic discovery processes (82).

Matrix effects were first described in detail by Kebarle and Tang in 1993 (75, 83). The authors observed a phenomenon in which the responses of organic bases were decreased when the presence of other organic bases was increased, which led to the hypothesis that coeluting, undetected matrix components were competing with the analyte for ionization when using electrospray ionization (75, 83). Electrospray ionization is the most commonly used type of ionization source in LC-MS (75). For successful ionization, the transfer of analyte from the liquid to the gas phase must occur and the analyte must become charged. Singly or multiply charged ions are produced in the electrospray interface by creating a fine spray of charged droplets in the presence of a strong electric field and heat (75, 84). Droplets are then evaporated, and analyte ions are directed through the mass spectrometer for detection. Incomplete evaporation of the droplet can result in non-transfer of analyte ions to the source, as conversion to the gas phase was incomplete (83, 84). This can occur when the analyte precipitates from the droplet solution. This was further demonstrated by King et al. who postulated that matrix effects are a result of nonvolatile matrix components and analyte ions compete for charge at the droplet surface, which subsequently allows them to be transferred to the gas phase (75, 84). The exact mechanism of reduction in release of analyte to the gas phase in the presence of nonvolatile species is unclear in these studies, but the authors hypothesize that the prevention of droplets to be reduced to smaller droplets could be a possible reason for ion suppression (75, 84). Trufelli et al. described the reasons for decreases in ionization efficiency as 1) the competition of

analyte and coeluting interference for access to droplet surface charge, 2) reduced droplet formation due to increased viscosity and surface tension, 3) formation of precipitating particles with non-volatile mobile phases additives, and 4) the formation of ion pairs between analyte and interferences or additives (85). Both ion suppression and ion enhancement are highly dependent on the matrix as well as the ionization source itself, and these effects can also be compound dependent (75, 86). Compounds with high mass and increased basicity more likely to cause matrix effects (87). Endogenous interferences can result from a plethora of species including lipids, salts, carbohydrates, ionic species, and highly polar compounds (88). Addition interferences can arise from metabolites, impurities, formulation agents, and degradation products that coelute with the analyte (77, 86). Polar compounds are more likely to suffer from matrix effects than nonpolar compounds, and species such as phospholipids are especially problematic when present in high concentrations (81). The removal of nonvolatile species can greatly reduce the potential for ion suppression (84). Other sources of matrix effects can include species from specimen containers as well as the types preservatives and anticoagulants that are present in some of these containers (75). Atmospheric pressure chemical ionization (APCI) techniques have been shown to be less susceptible to matrix effects and other interferences than ESI techniques (75, 76, 78, 82). One limitation of using APCI is that the analytes must be thermally stable for ionization (75). Despite ESI techniques having a higher probability for falling victim to these effects, they are still commonly used over APCI due to their increased sensitivity (77).

Matrix effects are generally studied during method development, optimization and validation using fortified matrices that often have a homogenous nature. However, this

does not account for the fact there may be variability between subjects or specimens. To overcome this challenge, it has been proposed that matrix effects should be evaluated in pooled matrices that originate from varying sources (75, 76). The two main techniques for assessing matrix effects are post-extraction addition and post-column (syringe) infusion (75, 76, 78, 79, 86). Post-extraction addition is a technique in which matrix effects are quantitatively determined for the analyte of interest. Analyte peak areas are determined for neat samples containing no matrix and compared to those of blank matrix samples that are fortified with analyte after extraction. The difference in analytical responses are presented as a percent difference from the neat standards with no matrix (100% signal) and the resulting signal in the presence of matrix. A calculated value of 0% represents no matrix effects. In this way, the matrix effect for several different samples can be statistically compared. One disadvantage of the post-extraction addition technique is that it only provides matrix effect estimation at the retention time of the analyte and thus is considered a static technique (75, 78). Conversely, post-column infusion is considered a dynamic technique in which analyte in constantly infused into the ion source with an infusion pump creating a constant detector signal. Drug-free matrix is simultaneously injected into the instrument and is separated using the chromatographic conditions of the method, then ionized along with the infused analyte. As such, the matrix effects can be visualized over an entire chromatographic run by assessing the changes in analyte response at different retention times (75, 78). Using this information, the retention of an analyte can be changed to avoid a problematic region in the chromatography. These post-column experiments should use an infusion of analyte that is within the analytical range of the method being assessed. Post-column infusion can allow for easier visualization of where enhancement or

suppression occurs using different extraction protocols, analytical columns, or mobile phases (75). Post-column infusion techniques are well-suited during method development and optimization, when chromatographic conditions can still be modified if problems arise. Quantitative analytical methods should be validated using these methods and should demonstrate that the method is free from matrix and metabolite interferences, other coeluting compounds, and is free from "cross-talk" effects when analytes may share product ions (76). While there are guidelines on the quantitative result of matrix effect evaluation (± 25% matrix effects, <15% CV), there are no qualitative guidelines for the assessment of these interferences besides the requirement that the change in baseline signal must not exceed 25% (50, 78). In best practice, both methods should be employed to gain a thorough assessment of matrix effects in analytical methods so that they can be mitigated as much as possible.

While coeluting compounds may cause matrix effects, there are certain measures that can be taken in an attempt to reduce these effects. Matrix effects can be affected by the extraction technique that is used and the chromatographic separation employed (i.e. elution profile, mobile phase, and analytical column) (78, 80, 86). The two approaches that are most commonly taken for mitigating matrix effects are to either modify sample preparation to reduce the number of interferences or to alter the chromatography to prevent coelution of analytes and endogenous species (75). Sample preparation can have a major impact on downstream interferences using LC-MS. Three major extraction techniques exist in forensic toxicology laboratories which are solid-phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation. Of the extraction techniques commonly used in forensic toxicology and other applications, simple protein precipitation is often

considered the most unclean technique, especially compared to LLE or SPE. Using multiple sample preparation steps may help rid methods of some matrix interferences (89).

Cleanliness is not the only factor that must be considered but also chromatographic separation when optimizing methods. Flow splitting can also be used to reduce matrix effects, as less solvent requires less ionization and may help mitigate ionization efficiency effects (75, 86). Another option for mitigating matrix interferences is by use of two-dimensional chromatography. Most matrix effects occur during the solvent front portion of the chromatographic run. In these cases, increasing the retention time of the analytes could be one way to reduce matrix effects. However, increases in the total run time resulting from the shift in chromatography can hinder high-throughput analysis. The use of "ballistic gradient" is another option for separating analytes and the solvent front which uses a rapid gradient to quickly elute the unwanted species at the beginning of the run (75, 85). Strongly retained endogenous species can remain on the analytical column and slowly elute causing ionization drift and increased background noise (81). The implementation of post-equilibration times or changes in gradient at the end of sample runs must be considered to eliminate some of these carryover effects.

A common approach aside from revisiting sample clean-up and chromatography is careful internal standard selection. Matrix effects and other interferences can lead to both positive and negative quantitative bias depending on if the interference affects ionization of the analyte or the internal standard. The overall analytical response may be monitored for an internal standard between a quality control and an extracted sample which can help identify decreased or enhanced ionization, but this is not true for samples where an analyte's concentration is unknown. As a result, an interference may go undetected or be

invisible to the analyst. The best approach for overcoming this analytical challenge is by using stable-isotope labeled internal standards (SIL-IS) (88, 90). These internal standards are a structural analog of the analyte of interest in which certain atoms (usually ^{1}H or ^{12}C) are replaced with isotopes (i.e. ^{2}H or ^{13}C). The isotopically labeled internal standard will maintain structural characteristics and behave almost identically to the analyte during various phases of method development (extraction and separation) (79, 91). Since the compounds will co-elute, any matrix effect that is observed will affect both the internal standard and the analyte and the relative response between them will remain unchanged, compensating for the interference (86). SIL-ISs are the gold standard for mitigating interferences in LC-MS based methods, but they are often not available for novel compounds as they require synthesis and can be quite costly. Higher costs of isotopically labeled internal standards may not be feasible for methods containing numerous compounds, so multiple analytes may share the same internal standard which cannot compensate for matrix effects of all analytes (88, 92). Although their use is ideal, ways to mitigate interferences in the absence of these labeled standards should be considered. Standard addition is a technique commonly used in laboratories, but its applicability in forensic toxicology may be limited. In this technique, a sample is fortified with known concentrations of a standard at increasing concentrations to form a calibration curve that can be used to extrapolate the unknown analyte concentration (88). For calibrator preparation this technique requires larger sample volumes, which are unfortunately often not available in forensic casework making this method a less popular option.

Manipulation of the mass spectrometric conditions may provide some alleviation of ion suppression and enhancement. The effects of suppression and enhancement may be

altered by switching from positive to negative electrospray ionization if a compound if amenable to both (79, 85). The orientation of the ionization source itself can influence the amount of suppression observed. It has been shown that linearly oriented sources are more prone to ion suppression than those with an orthogonal or Z-spray setup (80, 85, 93). In electrospray ionization, a solvent is pneumatically forced through a highly charged metal capillary, and the distance between the tip of this capillary (spray head) and the skimmer cone can be adjusted. This spray head position along with parameters such as capillary voltage can affect spray stability and subsequently the efficiency of ionization of the solvent and signal intensity (93). Other parameters that can affect the efficiency of ionization include the sheath and drying gas temperatures, as well as gas flow rates (93). Gas flow rates have been known to alter instrument response, and flow of nitrogen gas is used to protect the sample cone during sample transition from the ESI source and the mass analyzer. Although sheath gas (cone gas) flow does not have as pronounced of an effect, dramatic changes in ionization efficiency can be observed when the drying gas flow is reduced suggesting that desolvation is highly dependent on these conditions. Changing the gas temperature and flow rates can potentially reduce ion suppression (93). Similarly, decreases in sheath gas temperature can cause greater decreases in ion efficiency the farther away the capillary is placed from the aperture, meaning decreased desolvation can cause ions to not traverse the entire capillary plane (93). Although steps can be taken to attempt interference mitigation by altering these parameters, the special effects of ESI are lesser understood than other strategies so are described with less frequency.

Matrix effects have been described as “the Achilles heel” of LC techniques that use ESI (75). LC-MS/MS has become one of the most powerful tools in the pharmaceutical

industry due to its selectivity, sensitivity, and general applicability in modern quantitative techniques. However, this technique is susceptible to factors that can reduce ionization and therefore analytical response. While the underlying factors that decrease ionization efficiency may be reproducible between samples, it can ultimately lead to decreased sensitivity of methods (75). These issues are of significant concern when multiple analytes are being analyzed at once and in rapid LC method where the occurrence of analyte coelution is unavoidable. Method may suffer from significant ion suppression when the magnitude of interferent concentration well exceeds that of the target analyte. Therefore, novel ways to mitigate matrix effects and other co-extractive interferences in forensic toxicology casework is of the utmost concern for reliable and robust analysis, particularly in cases that utilize multi-analyte procedures in complex matrices.

Statement of the Problem

Suvorexant is a novel drug for the treatment of insomnia that has not been widely reported in forensic investigations. As a sedative hypnotic, suvorexant is expected to feature prominently in toxicology investigations, as with other therapeutics within this class such as zolpidem (Ambien®). The limited number of reports might be attributed to the scope of current toxicological testing and difficulties associated with its detection using routine immunoassay or GC/MS screening. Laboratories will need to consider suvorexant as a potentially impairing or toxic substance in both antemortem casework (e.g. impaired driving, DFSA) and medicolegal death investigations. Few methods have been published that describe suvorexant detection in biological samples. With suvorexant peak plasma concentrations expected to be less than 200 ng/mL with the most commonly prescribed 10-mg dose, sensitive analytical methods are needed for its detection in a variety of specimens

that are encountered in forensic toxicology. Limited research is available detailing suvorexant analysis in authentic specimens, but more cases may be reported with the increased availability of techniques that labs may easily adapt to their scope of testing.

No analytical methods were available describing the analysis in suvorexant in whole blood using high resolution mass-spectrometry (HRMS) techniques, and we are the first to describe a validated method. As a novel substance, the methods that are available for suvorexant describe analysis in the absence of a stable isotope internal standard (SILS-IS) which may pose challenges regarding matrix effects and drug interferences. While the previous methods described were free from matrix effects or interferences from a limited number of common analytes, developing a method that is impervious to interferences from coeluting compounds is nearly impossible in the absence of a SILS-IS. Other gaps in the literature for suvorexant are the limited metabolism studies that are available and the lack of commercially available metabolites for use in detection and method validation. The potential for suvorexant to undergo postmortem redistribution is relatively unknown, as only one study has been published that examined suvorexant concentrations in postmortem specimens and no remarkable pattern was identified. Many physicochemical properties of suvorexant have yet to be investigated or reported which could help aid in the interpretation of suvorexant concentrations in antemortem and postmortem casework.

The research described herein aims to develop analytical methods to improve the understanding of suvorexant properties that will aid forensic toxicologists in identifying and interpreting concentrations of the drug and its metabolites in forensic casework. The first objective of this research was to develop a highly sensitive and specific assay for the identification of suvorexant in whole blood using LC-Q/TOF-MS. Next, as LC-MS/MS is

also a commonly used platform in forensic toxicology, we describe the validation of an analytical method for the detection of suvorexant using LC-MS/MS. Performance of the LC-Q/TOF-MS and LC-MS/MS method were compared, specifically in regard to matrix effects and other interferences. To help understand the physicochemical properties of suvorexant, partition coefficients for the compound were determined both theoretically and experimentally over a range of ionic strength and pH to determine its lipophilicity and help assess its potential to undergo postmortem redistribution. Suvorexant was then detected in a variety of authentic case specimens using the developed and validated LC-Q/TOF-MS method to try to help characterize its presence in biofluids of forensic interest, and to compare to values obtained during the lipophilicity study. Due to the novelty of the drug, and the concern for capacity limited ionization using ESI techniques in LC-MS/MS, mitigation strategies for drug-mediated interferences were evaluated to help understand ways to overcome these challenges in the forensic laboratory. Finally, suvorexant biotransformations *in vitro* using cytochrome P450 recombinant enzymes were studied in order to optimize a method to identify metabolites using LC-Q/TOF-MS. The *in vitro* generated metabolite controls were used to qualitatively identify metabolites in authentic case specimens based on retention time matching, structural elucidation, and mass accuracy. The research described in this dissertation provides further insight regarding the identification, characterization, and metabolism of suvorexant in forensic case samples and implications it may have in forensic toxicology.

References

1. Citrome, L. (2014) Suvorexant for insomnia: a systematic review of the efficacy and safety profile for this newly approved hypnotic – what is the number needed to treat, number needed to harm and likelihood to be helped or harmed? *International Journal of Clinical Practice.* **68**, 1429-1441.

2. National Institutes of Health (2005) NIH State-of-the-Science Conference Statement on manifestations and management of chronic insomnia in adults. *NIH Consensus and State-of-the-Science Statements.* **22**, 1-30. Available from: https://consensus.nih.gov/2005/insomniastatement.pdf (accessed November 17, 2018).

3. Cox, C.D., Breslin, M.J., Whitman, D.B., Schreier, J.D., McGaughey, G.B., Bogusky, M.J., et al. (2010) Discovery of the Dual Orexin Receptor Antagonist [(7R)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl]methanone (MK-4305) for the Treatment of Insomnia. *Journal of Medicinal Chemistry.* **53**, 5320-5332.

4. Bennett, T., Bray, D. and Neville, M.W. (2014) Suvorexant, a dual orexin receptor antagonist for the management of insomnia. *P & T: A Peer-Reviewed Journal for Formulary Management.* **39**, 264-266.

5. Hoyer, D. and Jacobson, L.H. (2013) Orexin in sleep, addiction and more: Is the perfect insomnia drug at hand? *Neuropeptides.* **47**, 477-488.

6. Atkin, T., Comai, S. and Gobbi, G. (2018) Drugs for Insomnia beyond Benzodiazepines: Pharmacology, Clinical Applications, and Discovery. *Pharmacological Reviews.* **70**, 197.

7. Gotter, A.L., Winrow, C.J., Brunner, J., Garson, S.L., Fox, S.V., Binns, J., et al. (2013) The duration of sleep promoting efficacy by dual orexin receptor antagonists is dependent upon receptor occupancy threshold. *BMC Neuroscience.* **14**, 90.

8. Rhyne, D.N. and Anderson, S.L. (2015) Suvorexant in insomnia: efficacy, safety and place in therapy. *Therapeutic Advances in Drug Safety.* **6**, 189-195.

9. Born, S., Gauvin, D.V., Mukherjee, S. and Briscoe, R. (2017) Preclinical assessment of the abuse potential of the orexin receptor antagonist, suvorexant. *Regulatory Toxicology and Pharmacology.* **86**, 181-192.

10. Coleman, P.J., Gotter, A.L., Herring, W.J., Winrow, C.J. and Renger, J.J. (2017) The Discovery of Suvorexant, the First Orexin Receptor Drug for Insomnia. *Annual Review of Pharmacology and Toxicology.* **57**, 509-533.

11. Norman, J.L. and Anderson, S.L. (2016) Novel class of medications, orexin receptor antagonists, in the treatment of insomnia - critical appraisal of suvorexant. *Nature and Science of Sleep,* **8**, 239-247.

12. Herring, W.J., Roth, T., Krystal, A.D. and Michelson, D. (2019) Orexin receptor antagonists for the treatment of insomnia and potential treatment of other neuropsychiatric indications. *Journal of Sleep Research.* **28**, e12782.

13. Zarrabian, S., Riahi, E., Karimi, S., Razavi, Y. and Haghparast, A. (2018) The potential role of the orexin reward system in future treatments for opioid drug abuse. *Brain Research.* 146028.

14. Fujimoto, T., Kunitomo, J., Tomata, Y., Nishiyama, K., Nakashima, M., Hirozane, M., et al. (2011) Discovery of potent, selective, orally active benzoxazepine-based Orexin-2 receptor antagonists. *Bioorganic & Medicinal Chemistry Letters.* **21**, 6414-6416.

15. Callander, G., Olorunda, M., Monna, D., Schuepbach, E., Langenegger, D., Betschart, C., et al. (2013) Kinetic properties of "dual" orexin receptor antagonists at OX1R and OX2R orexin receptors. *Frontiers in Neuroscience.* **7,** 230.

16. Herring, W.J., Snyder, E., Budd, K., Hutzelmann, J., Snavely, D., Liu, K., et al. (2012) Orexin receptor antagonism for treatment of insomnia: a randomized clinical trial of suvorexant. *Neurology.* **79**, 2265-2274.

17. Gentile, T.A., Simmons, S.J., Barker, D.J., Shaw, J.K., España, R.A. and Muschamp, J.W. (2018) Suvorexant, an orexin/hypocretin receptor antagonist, attenuates motivational and hedonic properties of cocaine. *Addiction Biology.* **23**, 247-255.

18. Sakurai, T. (2013) Orexin deficiency and narcolepsy. *Current Opinion in Neurobiology.* **23**, 760-766.

19. Winrow, C.J., Gotter, A.L., Cox, C.D., Doran, S.M., Tannenbaum, P.L., Breslin, M.J., et al. (2011) Promotion of Sleep by Suvorexant--A Novel Dual Orexin Receptor Antagonist. *Journal of Neurogenetics.* **25**, 52-61.

20. Janto, K., Prichard, J.R. and Pusalavidyasagar, S. (2018) An Update on Dual Orexin Receptor Antagonists and Their Potential Role in Insomnia Therapeutics. *Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine.* **14**, 1399-1408.

21. Beuckmann, C.T., Ueno, T., Nakagawa, M., Suzuki, M. and Akasofu, S. (2019) Preclinical in vivo characterization of lemborexant (E2006), a novel dual orexin receptor antagonist for sleep/wake regulation. *Sleep.* **42**.

22. Ciu, D., Cabalu, T., Lai Yee, K., Small, J., Li, X., Liu, B., et al. (2016) *In vitro* and *in vivo* characterisation of the metabolism and disposition of suvorexant in humans. *Xenobiotica.* **46**, 1-14.

23. Sun, H., Yee, K.L., Gill, S., Liu, W., Li, X., Panebianco, D., et al. (2015) Psychomotor effects, pharmacokinetics and safety of the orexin receptor antagonist suvorexant administered in combination with alcohol in healthy subjects. *Journal of Psychopharmacology.* **29**, 1159-1169.

24. Yang, L. (2014) Suvorexant: First Global Approval. *Drugs.* **74**, 1817-1822.

25. Merck & Co. (2014). *Suvorexant Prescribing Information*. Available from: http://www.merck.com/product/usa/pi_circulars/b/belsomra/belsomra_pi.pdf (Accessed August 17, 2019).

26. Chen, Y., Zhou, Y., Li, J.-H., Sun, J.-Q. and Zhang, G.-S. (2015) Facile synthesis of suvorexant, an orexin receptor antagonist, via a chiral diazepane intermediate. *Chinese Chemical Letters.* **26**, 103-107.

27. Strotman, N.A., Baxter, C.A., Brands, K.M.J., Cleator, E., Krska, S.W., Reamer, R.A., et al. (2011) Reaction Development and Mechanistic Study of a Ruthenium Catalyzed Intramolecular Asymmetric Reductive Amination en Route to the Dual Orexin Inhibitor Suvorexant (MK-4305). *Journal of the American Chemical Society.* **133**, 8362-8371.

28. Mangion, I.K., Sherry, B.D., Yin, J. and Fleitz, F.J. (2012) Enantioselective Synthesis of a Dual Orexin Receptor Antagonist. *Organic Letters.* **14**, 3458-3461.

29. U.S. Drug Enforcement Administration (2014). Schedules of Controlled Substances: Placement of Suvorexant into Schedule IV. Final Rule. *Federal Register.* **79**, 51243-51247.

30. Sun, H., Kennedy, W.P., Wilbraham, D., Lewis, N., Calder, N., Li, X., et al. (2013) Effects of suvorexant, an orexin receptor antagonist, on sleep parameters as measured by polysomnography in healthy men. *Sleep.* **36**, 259-267.

31. Herring, W.J., Connor, K.M., Ivgy-May, N., Snyder, E., Liu, K., Snavely, D.B., et al. (2016) Suvorexant in Patients With Insomnia: Results From Two 3-Month Randomized Controlled Clinical Trials. *Biological Psychiatry.* **79**, 136-148.

32. U.S. Food and Administration (2013) Suvorexant Tablets, Insomnia Indication, Peripheral & Central Nervous System Drugs Advisory Committee Meeting. NDA 204569. Available from: https://wayback.archiveit.org/7993/20170405225048/https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCent

ralNervousSystemDrugsAdvisoryCommittee/UCM352970.pdf (accessed November 17, 2018).

33. Kripke, D. (2015) Is suvorexant a better choice than alternative hypnotics? *F1000Research*. **4**, 456.

34. Keks, N.A., Hope, J. and Keogh, S. (2017) Suvorexant: scientifically interesting, utility uncertain. *Australasian Psychiatry*. **25**, 622-624.

35. Michelson, D., Snyder, E., Paradis, E., Chengan-Liu, M., Snavely, D.B., Hutzelmann, J., et al. (2014) Safety and efficacy of suvorexant during 1-year treatment of insomnia with subsequent abrupt treatment discontinuation: a phase 3 randomised, double-blind, placebo-controlled trial. *The Lancet Neurology*. **13**, 461-471.

36. Sun, H., Palcza, J., Rosenberg, R., Kryger, M., Siringhaus, T., Rowe, J., et al. (2015) Effects of suvorexant, an orexin receptor antagonist, on breathing during sleep in patients with chronic obstructive pulmonary disease. *Respiratory Medicine*. **109**, 416-426.

37. Uemura, N., McCrea, J., Sun, H., Donikyan, M., Zammit, G., Liu, R., et al. (2015) Effects of the orexin receptor antagonist suvorexant on respiration during sleep in healthy subjects. *The Journal of Clinical Pharmacology*. **55**, 1093-1100.

38. Sun, H., Palcza, J., Card, D., Gipson, A., Rosenberg, R., Kryger, M., et al. (2016). Effects of suvorexant, an orexin receptor antagonist, on respiration during sleep in patients with obstructive sleep apnea. *Journal of clinical sleep medicine: JCSM: official publication of the American Academy of Sleep Medicine*. **12**, 9-17.

39. Patel, K.V., Aspesi, A.V., Evoy, K.E. (2015) Suvorexant: a dual orexin receptor antagonist for the treatment of sleep onset and sleep maintenance insomnia. *Annals of Pharmacotherapy*. **49**, 477-483.

40. Vermeeren, A., Sun, H., Vuurman, E.F.P.M., Jongen, S., Van Leeuwen, C.J., Van Oers, A.C.M., et al. (2015) On-the-road driving performance the morning after bedtime use of suvorexant 20 and 40 mg: A study in non-elderly healthy volunteers. *Sleep*. **38**, 1803-1813.

41. Vermeeren, A., Vets, E., Vuurman, E.F.P.M., Van Oers, A.C.M., Jongen, S., Laethem, T., et al. (2016) On-the-road driving performance the morning after bedtime use of suvorexant 15 and 30 mg in healthy elderly. *Psychopharmacology*. **233**, 3341-3351.

42. Breidinger, S.A., Simpson, R.C., Mangin, E. and Woolf, E.J. (2015) Determination of suvorexant in human plasma using 96-well liquid–liquid extraction and HPLC with tandem mass spectrometric detection. *Journal of Chromatography B*. **1002**, 254-259.

43. Carson, M. and Kerrigan, S. (2017) Quantification of suvorexant in urine using gas chromatography/mass spectrometry. *Journal of Chromatography B*. **1040**, 289-294.

44. Sullinger, S., Bryand, K. and Kerrigan, S. (2017) Identification of suvorexant in urine using liquid chromatography-quadrupole/time-of-flight mass spectrometry (LC-Q/TOF-MS). *Journal of Analytical Toxicology*. **41**, 224-229.

45. Iqbal, M., Ezzeldin, E., Khalil, N.Y., Al-Rashood, S.T.A. and Al-Rashood, K.A. (2017) Simple and highly sensitive UPLC-ESI-MS/MS assay for rapid determination of suvorexant in plasma. *Journal of Analytical Toxicology*. **41**, 114-120.

46. Iqbal, M., Khalil, N.Y., Ezzeldin, E. and Al-Rashood, K.A. (2018) Simultaneous detection and quantification of three novel prescription drugs of abuse (suvorexant, lorcaserin and brivaracetam) in human plasma by UPLC–MS-MS. *Journal of Analytical Toxicology*. **43**, 203-211.

47. Iqbal, M., Ezzeldin, E., Khalil, N.Y., Alam, P. and Al-Rashood, K.A. (2019) UPLC-MS/MS determination of suvorexant in urine by a simplified dispersive liquid-liquid micro-extraction followed by ultrasound assisted back extraction from solidified floating organic droplets. *Journal of Pharmaceutical and Biomedical Analysis*. **164**, 1-8.

48. Waters, B., Hara, K., Ikematsu, N., Takayama, M., Matsusue, A., Kashiwagi, M., et al. (2018) Tissue distribution of suvorexant in Three Forensic Autopsy Cases. *Journal of Analytical Toxicology*. **42**, 276-283.

49. ASB/ANSI 098 Standard for Mass Spectral Data Acceptance in Forensic Toxicology- Draft (2019).

    Available from:

    https://www.nist.gov/sites/default/files/documents/2019/03/20/standard_for_mass_spec_spectral_data_acceptance_-_asb.pdf (accessed June 17, 2019).

50. Scientific Working Group for Forensic Toxicology (2013) Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. *Journal of Analytical Toxicology*. **37**, 452-474.
51. Asha, S. and Vidyavathi, M. (2010) Role of human liver microsomes in in vitro metabolism of drugs-a review. *Applied Biochemistry and Biotechnology*. **160**, 1699-1722.
52. Anzenbacher, P. and Anzenbacherova, E. (2001) Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences*. **58**, 737-747.
53. Iwatsubo, T., Hirota, N., Ooie, T., Suzuki, H., Shimada, N., Chiba, K., et al. (1997) Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. *Pharmacology and Therapeutics*. **73**, 147-171.
54. Li, F., Gonzalez, F.J. and Ma, X. (2012) LC–MS-based metabolomics in profiling of drug metabolism and bioactivation. *Acta Pharmaceutica Sinica B*. **2**, 118-125.
55. Lai, F.Y., Erratico, C., Kinyua, J., Mueller, J.F., Covaci, A. and van Nuijs, A.L.N. (2015) Liquid chromatography-quadrupole time-of-flight mass spectrometry for screening in vitro drug metabolites in humans: investigation on seven phenethylamine-based designer drugs. *Journal of Pharmaceutical and Biomedical Analysis*. **114** (Supplement C), 355-375.
56. Manier, S.K., Keller, A., Schäper, J. and Meyer, M.R. (2019) Untargeted metabolomics by high resolution mass spectrometry coupled to normal and reversed phase liquid chromatography as a tool to study the in vitro biotransformation of new psychoactive substances. *Scientific Reports*,.**9**, 2741.

57. Cook, D.S., Braithwaite, R.A. and Hale, K.A. (2000) Estimating antemortem drug concentrations from postmortem blood samples: the influence of postmortem redistribution. *Journal of Clinical Pathology*. **53**, 282.

58. Skopp, G. (2010). Postmortem toxicology. *Forensic Science, Medicine, and Pathology.* **6**, 314-325.

59. Pélissier-Alicot, A.-L., Gaulier, J.-M., Champsaur, P. and Marquet, P. (2003) Mechanisms Underlying Postmortem Redistribution of Drugs: A Review. *Journal of Analytical Toxicology*. **27**, 533-544.

60. Zilg, B., Thelander, G., Giebe, B. and Druid, H. (2017) Postmortem blood sampling—Comparison of drug concentrations at different sample sites. *Forensic Science International*. **278**, 296-303.

61. Jones, G.R. and Pounder, D.J. (1987) Site Dependence of Drug Concentrations in Postmortem Blood—A Case Study*. *Journal of Analytical Toxicology*. **11**, 186-190.

62. Drummer, O.H. (2004) Postmortem toxicology of drugs of abuse. *Forensic Science International*. **142**, 101-113.

63. Baselt, R.C. (2017) Suvorexant. In Disposition of toxic drugs and chemicals in man, 11th edition. Biomedical Publications, Seal Beach, CA, pp. 2005.

64. Andrés, A., Rosés, M., Ràfols, C., Bosch, E., Espinosa, S., Segarra, V., et al. (2015) Setup and validation of shake-flask procedures for the determination of partition coefficients (logD) from low drug amounts. *European Journal of Pharmaceutical Sciences.* **76,** 181-191.

65. Kah, M. and Brown, C.D. (2008) LogD: lipophilicity for ionisable compounds. *Chemosphere*. **72**, 1401-1408.

66. Donovan, S.F. and Pescatore, M.C. (2002) Method for measuring the logarithm of the octanol–water partition coefficient by using short octadecyl–poly(vinyl alcohol) high-performance liquid chromatography columns. *Journal of Chromatography A.* **952**, 47-61.

67. Pallicer, J.M., Calvet, C., Port, A., Rosés, M., Ràfols, C. and Bosch, E. (2012) Extension of the liquid chromatography/quantitative structure–property relationship method to assess the lipophilicity of neutral, acidic, basic and amphotheric drugs. *Journal of Chromatography A.* **1240**, 113-122.

68. Kempińska, D., Chmiel, T., Kot-Wasik, A., Mróz, A., Mazerska, Z. and Namieśnik, J. (2019) State of the art and prospects of methods for determination of lipophilicity of chemical compounds. *TrAC Trends in Analytical Chemistry*. **113,** 54-73.

69. Taka´cs-Nova´k, K. and Avdeel, A. (1996). Interlaboratory study of log P determination by shake-flask and potentiometric methods. *Journal of Pharmaceutical and Biomedical Analysis*. **14**, 1405-1413.

70. Chou, J.T. and Jurs, P.C. (1979) Computer-assisted computation of partition coefficients from molecular structures using fragment constants. *Journal of Chemical Information and Computer Sciences*. **19**, 172-178.

71. Medić-Šarić, M., Mornar, A., Badovinac-Črnjević, T., and Jasprica, I. (2004) Experimental and calculation procedures for molecular lipophilicity: A

comparative study for 3,3'-(2-Methoxybenzylidene)bis(4-hydroxycoumarin). *Croatica Chemica Acta.* **77**, 367-370.

72. Medic-Saric, M., Mornar, A. and Jasprica, I. (2004) Lipophilicity study of salicylamide. *Acta Pharmaceutica.* **54**, 91-101.
73. Spessard, G.O. (1998) ACD Labs/LogP dB 3.5 and ChemSketch 3.5. *Journal of Chemical Information and Computer Sciences.* **38**, 1250-1253.
74. Petrauskas, A. and Kolovanov, E. (2000) ACD/Log P method description. *Perspectives in Drug Discovery and Design.* **19**, 99-116.
75. Taylor, P. J. (2005) Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry*. **38**, 328-334.
76. Matuszewski, B.K., Constanzer, M.L. and Chavez-Eng, C.M. (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*. **75**, 3019-3030.
77. Larger, P.J., Breda, M., Fraier, D., Hughes, H. and James, C.A. (2005). Ion-suppression effects in liquid chromatography–tandem mass spectrometry due to a formulation agent, a case study in drug discovery bioanalysis. *Journal of Pharmaceutical and Biomedical Analysis*. **39**, 206-216.
78. Peters, F. T., and Remane, D. (2012) Aspects of matrix effects in applications of liquid chromatography–mass spectrometry to forensic and clinical toxicology—a review. *Analytical and Bioanalytical Chemistry*. **403**, 2155-2172.

79. Van Eeckhaut, A., Lanckmans, K., Sarre, S., Smolders, I. and Michotte, Y. (2009) Validation of bioanalytical LC–MS/MS assays: Evaluation of matrix effects. *Journal of Chromatography B*. **877**, 2198-2207.

80. Ghosh, C., Shinde, C. P., and Chakraborty, B. S. (2012) Influence of ionization source design on matrix effects during LC–ESI-MS/MS analysis. *Journal of Chromatography B*. **893-894**, 193-200.

81. Ismaiel, O.A., Zhang, T., Jenkins, R.G. and Karnes, H.T. (2010) Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry. *Journal of Chromatography B*. **878**, 3303-3316.

82. Fang, N., Yu, S., Ronis, M.J. and Badger, T.M. (2015). Matrix effects break the LC behavior rule for analytes in LC-MS/MS analysis of biological samples. *Experimental biology and medicine*. **240**, 488-497.

83. Kebarle, P., and Tang, L. (1993) From ions in solution to ions in the gas phase - the mechanism of electrospray mass spectrometry. *Analytical Chemistry*. **65**, 972A-986A.

84. King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C. and Olah, T. (2000) Mechanistic investigation of ionization suppression in electrospray ionization. *Journal of the American Society for Mass Spectrometry*. **11**, 942-950.

85. Trufelli, H., Palma, P., Famiglini, G., and Cappiello, A. (2011) An overview of matrix effects in liquid chromatography-mass spectrometry. *Mass Spectrometry Reviews*. **30**, 491-509.

86. Chambers, E., Wagrowski-Diehl, D. M., Lu, Z., and Mazzeo, J. R. (2007) Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography B.* **852**, 22-34.

87. Shaw, P.N., Tan, S.K. and Hewavitharana, A. (2014) Strategies for the detection and elimination of matrix effects in quantitative LC–MS analysis. *LC GC North America.* **32**, 54-64.

    Available from: http://www.chromatographyonline.com/strategies-detection-and-elimination-matrix-effects-quantitative-lc-ms-analysis (accessed October 11, 2019).

88. Furey, A., Moriarty, M., Bane, V., Kinsella, B., and Lehane, M. (2013) Ion suppression; A critical review on causes, evaluation, prevention and applications. *Talanta*. **115**, 104-122.

89. Rossmann, J., Gurke, R., Renner, L.D., Oertel, R. and Kirch, W. (2015) Evaluation of the matrix effect of different sample matrices for 33 pharmaceuticals by post-column infusion. *Journal of Chromatography B.* **1000**, 84-94.

90. Gosetti, F., Mazzucco, E., Zampieri, D. and Gennaro, M.C. (2010) Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A.* **1217**, 3929-3937.

91. Berg, T., and Strand, D. H. (2011) 13C labelled internal standards—A solution to minimize ion suppression effects in liquid chromatography–tandem mass spectrometry analyses of drugs in biological samples? *Journal of Chromatography A.* **1218**, 9366-9374.

92. Villagrasa, M., Guillamón, M., Eljarrat, E. and Barceló, D. (2007) Matrix effect in liquid chromatography–electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material. *Journal of Chromatography A*. **1157**, 108-114.

93. Janusson, E., Hesketh, A. V., Bamford, K. L., Hatlelid, K., Higgins, R., and McIndoe, J. S. (2015) Spatial effects on electrospray ionization response. *International Journal of Mass Spectrometry*. **388**, 1-8.

CHAPTER II

QUANTIFICATION OF SUVOREXANT IN BLOOD USING LIQUID CHROMATOGRAPHY-QUADRUPOLE/TIME OF FLIGHT (LC-Q/TOF) MASS SPECTROMETRY[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1] Skillman, B., and Kerrigan, S. (2018) Journal of Chromatography B. **1091**, 87-95. Reprinted with permission of the publisher.

Abstract

Suvorexant is a novel drug for the treatment of insomnia that is marketed under the trade name Belsomra®. Unlike other hypnotics, suvorexant is a dual orexin receptor antagonist that is believed to have a lower abuse potential compared to other therapeutics. Although sedative hypnotics feature prominently in forensic toxicology investigations, there have been limited reports that describe the analysis of suvorexant in biological samples. Following a 10-mg oral dose, peak concentrations are typically less than 200 ng/mL. A highly sensitive assay is required because forensic toxicology laboratories are often required to identify a drug several hours after a single dose. A new analytical procedure for the quantification of suvorexant in whole blood was developed that will aid in the identification of this new drug in forensic toxicology casework. A simple acidic/neutral liquid-liquid extraction (LLE) was used to isolate suvorexant from whole blood followed by liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry analysis using positive electrospray ionization (ESI). The extraction efficiencies of various solvents in blood were evaluated in addition to limit of detection, limit of quantitation, precision, accuracy and bias, calibration model, matrix effects, interferences, and carryover. The recovery of suvorexant was evaluated using four different extraction solvents (*N*-butyl chloride, ether/toluene (1:1), hexane/ethyl acetate (9:1), and methyl tert-butyl ether (MTBE)). Although no significant differences in analytical recovery were observed, *N*-butyl chloride demonstrated improved reproducibility, efficiency and convenience. A weighted (1/x) quadratic calibration model was selected over a range of 2-200 ng/mL (R^2=0.995). Using only 0.5 mL whole blood, limits of detection and quantification were 0.5 ng/mL. Intra-assay (n=5) and inter-assay (n=15) precision (% CV)

were ≤ 13% and bias ranged from -5 to 2% at concentrations of 5, 50, and 160 ng/mL. Matrix effects were 16% (9% CV) and 15% (8% CV) for 20 ng/mL and 100 ng/mL (n=20), respectively. No qualitative interferences or carryover were observed; however, a quantitative interference with the internal standard, estazolam-D5, could be attributed to sertraline when present at a 10-fold higher concentration. In the absence of a commercially available deuterated internal standard, the potential for quantitative interferences using LC-based methods are discussed.

Keywords: Suvorexant, LC-Q/TOF-MS, Blood, Forensic toxicology

QUANTIFICATION OF SUVOREXANT IN BLOOD USING LIQUID CHROMATOGRAPHY-QUADRUPOLE/TIME OF FLIGHT (LC-Q/TOF) MASS SPECTROMETRY

Introduction

Insomnia is a prevalent medical condition that affects approximately one-third of the adult population in America and can be described as difficulty falling asleep, staying asleep, or experiencing nonrestorative sleep (1,2). Insomnia has been associated with depression, anxiety disorders, irritability, inability to concentrate, and a general diminished quality of life (2). Most commonly, pharmacological interventions include the use of benzodiazepines and other drugs that modulate the gamma-amino butyric acid (GABA) receptor. However, these receptor agonist hypnotics can stimulate GABA and may pose consequences such as rebound insomnia, next-morning sedation, amnesia, potential for abuse, and physical dependence (3).

Suvorexant, also known as MK-4305, is a novel drug that is used for the treatment of insomnia (4). Suvorexant is marketed under the trade name Belsomra® and is manufactured by Merck & Co. as a dual orexin receptor antagonist (DORA). In August 2014, the Food and Drug Administration approved suvorexant and in February of 2015 it became commercially available. Currently, suvorexant is listed under Schedule IV of the Controlled Substances Act (5). Its mechanism of action is unique from other clinically approved hypnotic drugs, because it affects the activity of orexin neurons in the lateral hypothalamus, which are thought to play a key role in the regulation of wakefulness (6). As a dual orexin receptor antagonist, suvorexant blocks both OX_1R and OX_2R receptors which promote sleep by inhibiting orexin A and B. Thus, suvorexant aids in the transition

from wakefulness to sleep, and has no effect on GABA receptors. This drug provides alternative treatment options for insomnia, and is reported to have a lower potential for addiction compared to existing therapeutics (7).

Suvorexant should be administered within 30 min of going to sleep, and not<7 h of the time of awakening. The recommended oral dose of suvorexant is 10 mg, although doses of 15 and 20 mg are also available (3). The drug is primarily metabolized by cytochrome P450 CYP3A4 and CYP2C19 enzyme systems. The proposed metabolites of suvorexant are the M4 metabolite produced by carboxylation of the parent drug, and the M9 metabolite produced by hydroxylation (**Figure 1**) (8). Suvorexant metabolites are not yet commercially available, so their analysis is precluded at the present time.

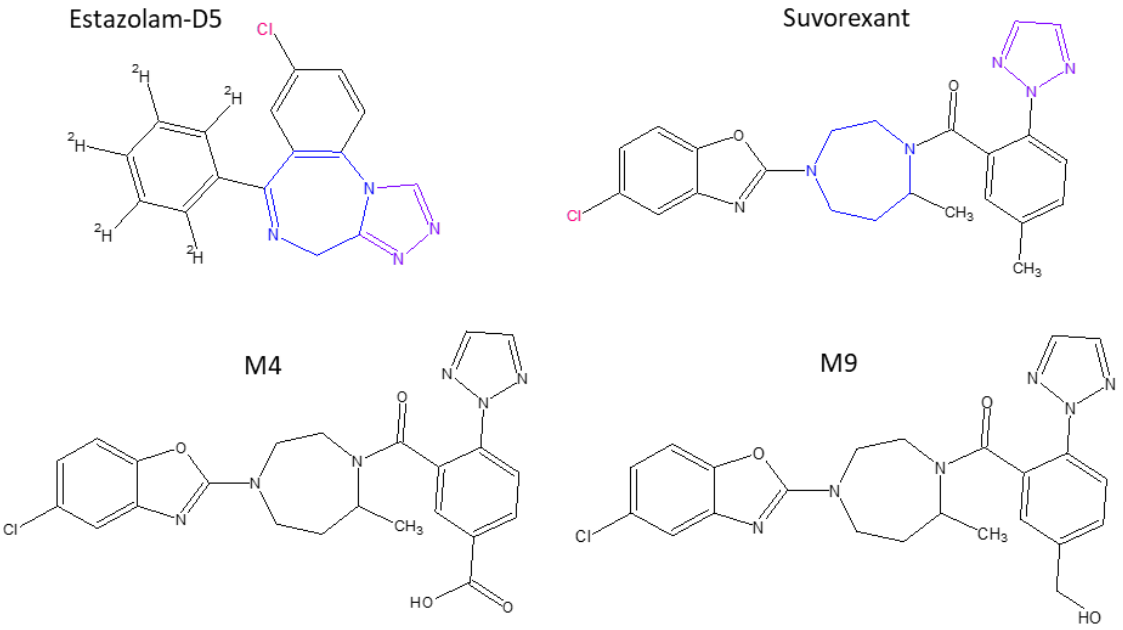


Figure 2.1. Chemical structures of suvorexant and its carboxylated and hydroxylated metabolites, M4 and M9, respectively. Estazolam-D5 was selected as the internal standard due to its structural similiarities to suvorexant. These include a 7-membered azepane/azepine ring, a heterocyclic triazole, and a chlorine.

Suvorexant is reported to be eliminated predominantly as inactive metabolite in feces. The drug is extensively protein-bound (99.5%), predominantly to α-1 acid glycoprotein and serum albumin (9). The half-life of suvorexant is approximately 12 h and steady-state plasma concentrations are reached within three days of daily administration (8). Peak plasma concentrations occur approximately two hours after administration on an empty stomach, but ingestion of suvorexant following a meal can delay the time to maximum concentration (T_{max}) by an additional 1.5 h (9). Although race and age do not seem to have an impact on peak plasma concentrations (C_{max}), they are reported to be higher in females by about 9%, and in obese patients by 17%. The oral bioavailability of suvorexant is reported to be approximately 82%, with absorption of the drug having an inversely proportional relationship to the dose administered, resulting in decreased bioavailability as the dose increases (10). Blood plasma ratios have yet to be reported and very little is known of its distribution in tissues and fluids of toxicological interest (11).

Generally, short half-lives and rapid clearance are preferred for hypnotic medications. The likelihood of residual effects, drowsiness or decreased alertness increases in a dose dependent fashion. These effects have the potential to interfere with daily activities (7). The FDA recommends that next-day activities, such as driving, be avoided by patients taking the maximum daily dose of 20 mg (4). Sedative hypnotics (such as zolpidem) feature prominently in impaired driving and drug-facilitated sexual assault investigations. The long half-life of the drug raises concerns that drivers may be impaired for extended periods following its use. However, most forensic toxicology laboratories do not routinely screen for suvorexant, so very little is understood regarding its prevalence or role in human performance toxicology investigations. Moreover, due to its high boiling

point (669 °C, 450.9 g/mol), suvorexant is a very late eluting compound using gas chromatography/mass spectrometry (GC/MS) techniques, which increases the likelihood that the drug might go undetected (12).

There are relatively few published reports that describe the quantitative analysis of suvorexant in biofluids of forensic significance. Merck & Co. published an analytical method for the detection of the drug in plasma using liquid-liquid extraction (LLE) and liquid chromatography- tandem mass spectrometry (LC-MS/MS) over a concentration range of 1–1000 ng/mL. This method utilized an isotopically labeled internal standard (suvorexant-$^{13}C^{2}H_{3}$) that was manufactured in-house by Merck (2). The method was used to quantify parent drug in plasma samples as part of the clinical study. However, the method was not validated in accordance with generally accepted standards in forensic toxicology (13). Additionally, this LC-MS/MS procedure utilized only one transition and did not utilize a secondary (qualifying ion), precluding the use of ion ratios for evaluation or acceptance purposes. This approach is not forensically defensible, since it is generally accepted that a minimum of two ions are required (14). More recently, Iqbal et al. developed a method for the determination of suvorexant in plasma using LLE and LC MS/MS using rivaroxaban as the internal standard. A linear concentration range of 0.33 200 ng/mL was used with an LOD of 0.1 ng/mL and LOQ of 0.33 ng/mL (15). Carson et al. reported a quantitative assay in urine using LLE and gas chromatography/ mass spectrometry (GC/MS). In the absence of a commercially available deuterated internal standard, estazolam-D5 was used due to its structural similarity to the compound of interest. These include a 7- membered azepine ring, heterocyclic triazole, and a chlorine moiety (**Figure 2.1**). Performance of the assay proposed by Carson et al. was evaluated in

accordance with the Scientific Working Group for Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology (13). The limits of detection and quantitation for the assay in urine were determined to be 10 ng/mL (16).

Similarly, a quadrupole time-of-flight liquid chromatography/mass spectrometry (LC-Q/TOF-MS) method in urine was developed and optimized by Sullinger et al. using a modified LLE procedure as previously described (12,16). Liquid-liquid extraction was previously identified as the preferred extraction technique due to suvorexant's high miscibility with organic solvents. The LOD for the method was determined to be 0.5 ng/mL and the LOQ was 5 ng/mL. Despite the lack of a deuterium or isotopically labeled internal standard for suvorexant, the assay demonstrated excellent precision and accuracy throughout the entire calibration range (2–250 ng/mL) (12).

The purpose of this study was to develop, optimize and validate a method for the detection and quantification of suvorexant in whole blood samples using LC-Q/TOF-MS. Blood is the most common matrix encountered in both death investigations (postmortem toxicology) and human performance toxicology. Since hypnotic drugs feature so prominently in both types of casework, and suvorexant is of particular importance from the standpoint of impaired driving and drug-facilitated sexual assault, forensic laboratories should have analytical methods that are capable of identifying this new drug.

Suvorexant was isolated from blood using a modified acidic/neutral liquid-liquid extraction. This approach should allow laboratories to adapt their existing acidic/neutral extraction protocols to identify suvorexant readily. In this study, extraction efficiencies in blood were further investigated and the method was validated in terms of limits of

detection, quantification, precision, bias, calibration model, matrix effects, interferences, carryover, and processed sample stability.

Materials and Methods

Chemicals and Reagents

Suvorexant ([(7R)-4-(5-chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3,-triazol-2-yl)phenyl]methanone) was purchased from Adooq Bioscience as a powder (Irvine, CA). Estazolam-D5 internal standard was purchased from Cerilliant Corp. (Round Rock, TX) at a concentration of 100 μg/mL in methanol. Fifty-three additional interfering drugs were purchased as 1 mg/mL methanolic standards from Cerilliant Corp. (Round Rock, TX) as follows: (+)-propoxyphene, 7-aminoclonazepam, 7-aminoflunitrazepam, acetaminophen, alprazolam, amitriptyline, amobarbital, amphetamine, bupropion, butalbital caffeine, carbamazepine, carisoprodol, clonazepam, cocaine, codeine, cyclobenzaprine, dextromethorphan, diazepam, fluoxetine, flurazepam, gabapentin, hydrocodone, hydromorphone, ketamine, methylenedioxymethamphetamine (MDMA), meperidine, meprobamate, methadone, methaqualone, morphine, nordiazepam, oxazepam, oxycodone, oxymorphone, pentobarbital, phencyclidine, phenobarbital, phenytoin, pseudoephedrine, salicylic acid, secobarbital, sertraline, temazepam, delta-9-tetrahydrocannabinol (THC), carboxy-THC, tramadol, trazodone, valproic acid, zaleplon, zolpidem and zopiclone.

Acetic acid (glacial) (ACS grade), sodium acetate (ACS grade), and toluene (ACS grade) were obtained from Mallinckrodt Chemicals (St. Louis, MO). LC/MS grade methanol and ACS grade ether were obtained from J.T. Baker (Center Valley, MA). Formic acid (LC/MS grade) and methyl tert-butyl ether (MTBE) were obtained from Sigma-

Aldrich (St. Louis, MO). *N*-butyl chloride (99+%, pure) manufactured by Acros Organics and LC/MS grade acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). A Millipore Direct-Q ® UV Water Purification System (Billerica, MA) was used for the purification of all deionized water.

Sodium acetate buffer (0.4 M) at pH 3.6 was prepared for routine use in extraction. Mobile phase A and B consisted of 0.1% formic acid in deionized water and in acetonitrile, respectively. Mobile phase A and B (1:1) were used for the reconstitution of analyte for injection into the instrument. Pooled drug-free bovine blood preserved with 1% sodium fluoride and 0.2% potassium oxalate was purchased from Quad Five (Ryegate, MT) and stored at 4 °C.

Suvorexant stock solution was prepared at a concentration of 0.1 mg/mL in methanol. Working standards were routinely prepared in methanol at concentrations 0.01, 0.1, 1, and 10 ng/μL. A working standard solution of estazolam-D5 was prepared in methanol at 2 ng/μL.

Instrumentation

The LC-Q/TOF-MS used was manufactured by Agilent Technologies (Santa Clara, CA) and consisted of a 1290 Infinity Binary LC System and a 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS system equipped with electrospray ionization (ESI) technology. Gradient elution was performed for the chromatographic separation of the compounds using a Poroshell EC-C18 column (2.1×100 mm, 2.7 μm particle size) and a Poroshell 120 EC-C18 guard column (2.1×5 mm, 2.7 μm particle size). The column temperature was maintained at 35 °C with a flow rate of 0.4 mL/min. The gradient elution

profile was 40–80% B (0–3 min), a 1 min hold, a decrease to 40% B (5 min), followed by re-equilibration.

The electrospray (ESI) probe apparatus was operated in positive mode. Ionization conditions were fully optimized as follows: 300 °C gas temperature, 13 L/min gas flow, 45 psi nebulizer pressure, 350 °C sheath gas temperature, and 12 L/min sheath gas flow. The mass spectrometer parameters were 3000 V capillary voltage, 2000 V nozzle voltage, and 150 V fragmentor voltage. The collision energies used were 50 eV for suvorexant and 30 eV for estazolam-D5. The MS scan rate was 10 spectra/s and the MS/MS scan rate was 5 spectra/s over a MS scan range of 100–1600 m/z. Using targeted MS/MS data acquisition, the two product ion transitions used for suvorexant and estazolam-D5 were m/z 451.1644 > 186.0664; m/z 451.1644 > 104.0493 and m/z 300.1059 > 272.0875; m/z 300.1059 > 210.1076, respectively (**Figure 2.2**).

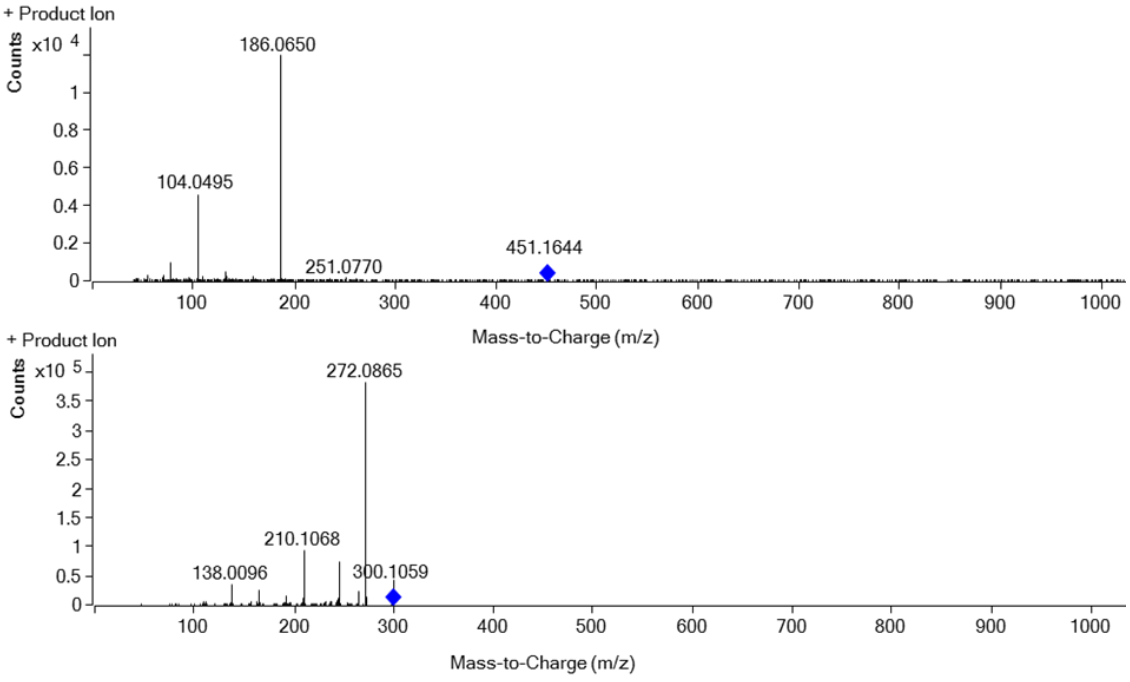


Figure 2.2. MS/MS spectra of suvorexant (upper) and estazolam-D5 (lower) at the optimum collision energy of 50 eV and 30 eV, respectively.

Extraction Optimization

Our previously published method for urine was adapted for use with whole blood (12). Calibrators and controls were prepared at 0, 2, 5, 10, 50, 100, and 250 ng/mL by fortifying 0.5 mL of blood with the appropriate volume of suvorexant working standard in 10 mL screw-top round bottom glass centrifuge tubes. Internal standard was added to achieve a final concentration of 100 ng/mL. Following fortification of the blood, 1 mL sodium acetate buffer (pH 3.6, 0.4 M) and 2.5 mL ether/toluene (1:1) were added, respectively. The samples were placed on a rotary mixer for 5 min, followed by centrifugation at 3000 rpm for 5 min. The organic layer was removed and placed into conical glass tubes, which was then evaporated to dryness under nitrogen (50 °C). The extracts were then reconstituted with 30 µL of mobile phase A and B (1:1) and 2 µL was injected into the LC-Q/TOF-MS.

Recovery was evaluated using four different extraction solvents as follows: ether/toluene (1:1), MTBE, *N*-butyl chloride, and hexane/ethyl acetate (9:1). In a 10 mL round bottom glass centrifuge tube, 0.5 mL of bovine blood was fortified with estazolam-D5 internal standard to achieve a final concentration of 100 ng/mL. For extracted samples, blood was then fortified with 100 ng/mL suvorexant prior to liquid-liquid extraction. To each tube 1 mL sodium acetate buffer (pH 3.6, 0.4 M) was added followed by 2.5 mL of the appropriate extraction solvent. Samples were placed on the rotary mixer for 5 min then centrifuged at 3000 rpm for 5 min. The organic phase was removed and placed into glass conical tubes, and non-extracted samples were fortified with suvorexant to reach a final concentration of 100 ng/mL. Extracts were evaporated to dryness under nitrogen at 50 °C, reconstituted with 30 µL of mobile phase A/B (1:1) and analyzed using LC-Q/ TOF-MS.

Samples extracted using methyl tert-butyl ether were not injected due to their visual appearance, which was dark in color. Recoveries were calculated by direct comparison of the relative peak areas (drug/IS) of extracted and non-extracted samples in quadruplicate.

Following selection of *N*-butyl chloride as the extraction solvent, the injection volume was optimized (2–10 μL). Using low calibrators (0.5, 1, 2, 5, and 10 ng/mL), peak shape, abundance and signal-to-noise (S/N) ratios were evaluated.

Method Validation

Assay performance was evaluated in accordance with SWGTOX recommendations to include extraction efficiency, limit of detection, limit of quantification, calibration model precision, accuracy and bias, carryover, processed sample stability, matrix effects, and interferences.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by fortification of three independent sources of blood with suvorexant and internal standard. Each of the three blood samples were analyzed in duplicate over three runs at each concentration (0.5, 1, 2, 5, and 10 ng/mL). The LOD was determined by selecting the lowest concentration to produce a retention time within 2% of the verified standard, ion ratios within 20%, and signal-to-noise ratio > 3:1. The LOQ was defined as lowest concentration to produce a retention time within 2% of the standard, ion ratios within 20%, a signal-to-noise ratio >10:1, and concentration within 20% of the expected concentration.

Calibration models were evaluated using eight non-zero calibrators in whole blood (2, 5, 10, 25, 50, 100, 150, and 200 ng/mL) and 100 ng/mL IS over five days. The calibrators were extracted using the previously described method and analyzed using LC-

Q/TOF-MS. Precision and bias were evaluated at low, medium, and high concentrations (5, 50, and 160 ng/mL) in pooled blood in triplicate over five days.

Carryover was evaluated by injecting drug-free extracts immediately following the highest suvorexant calibrator (200 ng/mL) in triplicate. Processed sample stability was evaluated using a single positive blood control (100 ng/mL suvorexant and IS). Quantitative measurements were made in triplicate following 0, 6, 18, 24, 48, and 60 h of storage in the autosampler compartment (4 °C).

Ion suppression or enhancement was evaluated qualitatively during method development using post column infusion. Ten drug-free blood samples from independent sources were extracted and injected onto the LC-Q/TOF-MS while suvorexant and internal standard were infused directly into the source using a syringe driver (KD Scientific, KDS 100 Legacy Single Syringe Pump, Hollistion, MA) and T-connector. Matrix effects were evaluated quantitatively using the post-extraction addition technique. Ten drug-free matrices were extracted in duplicate. Following evaporation, samples were fortified with a low and high concentration of suvorexant (20 ng/mL and 100 ng/mL) and internal standard (100 ng/mL). Mobile phase was fortified with the same concentrations of suvorexant and internal standard. Matrix effects were evaluated by comparing the abundance of drug and internal standard in the presence and absence of biological matrix Criteria for acceptability were < 25% matrix effect and CV < 15%.

Interferences from other drugs were determined by fortifying blood samples with the fifty-three common drugs listed previously. Drug interferences were evaluated using positive and negative controls of suvorexant and IS in the presence of other drugs (potential interferents) at a ten-fold and 100-fold higher concentration.

Results and Discussion

Extraction Optimization

The analytical recovery of suvorexant was evaluated using four different extraction solvents (**Figure 2.3**). Ether/toluene (1:1) had marginally higher recovery (93 ± 12%) but required extensive evaporation time due to the high boiling point of toluene. Although hexane/ethyl acetate (9:1) produced adequate recovery of suvorexant (82 ± 10%), the internal standard abundance was significantly reduced (**Figure 2.4**) and MTBE was not evaluated due to its visual (darkened) appearance. Although *N*-butyl chloride appeared to have slightly lower recovery (77 ± 6%), it showed improved reproducibility (**Figure 2.3**). ANOVA and a two-tailed student t-test were used to evaluate analytical recoveries of *N*-butyl chloride and ether/toluene. No significant difference was evident ($p=0.12$, $\alpha=0.05$), so *N*-butyl chloride was selected for convenience (faster evaporation) and reduced reagent preparation time. Optimum injection volumes were evaluated in terms of absolute peak area, peak shape, and signal-to-noise ratio. Although no qualitative differences were observed, adequate sensitivity and S/N ratios were achieved using a 2 μL injection (44:1 at 0.5 ng/mL).

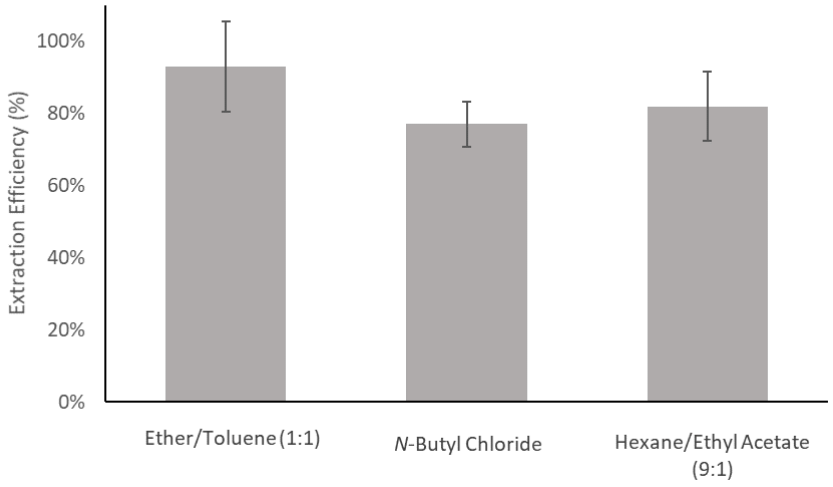


Figure 2.3. Extraction efficiency of suvorexant from whole blood using ether/toluene (1:1), *N*-butyl chloride, and hexane/ethyl acetate (9:1). Data represents the mean ± 1 SD.

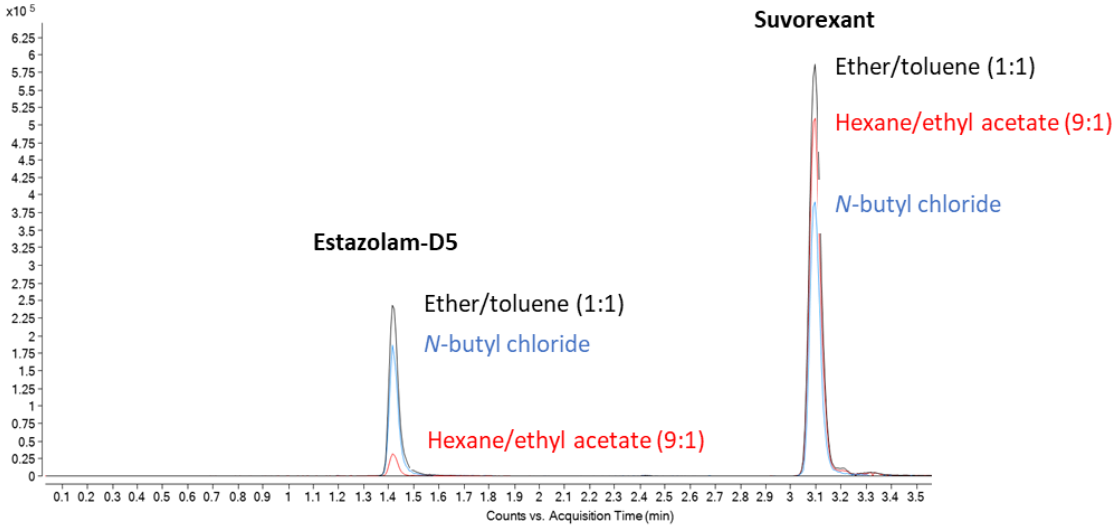


Figure 2.4. Total Ion Chromatograms (TICs) of the internal standard and suvorexant in blood (100 ng/mL) depicting the reduced recovery of estazolam-D5 using hexane/ethyl acetate (9:1) as the extraction solvent.

Method Validation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined empirically by fortification of whole blood with suvorexant over three days. The LOD and LOQ were determined to be 0.5 ng/mL. At the LOQ, the mean S/N was 47:1 and accuracy was 110%. The mean calculated concentrations, accuracy, precision (%CV) and S/N ratios (0.5–10 ng/mL) are summarized in **Table 2.1**. Examples of chromatographic quality of samples at both the LOD and LOQ are depicted with an extracted ion chromatogram in **Figure 2.5**.

Table 2.1. Quantitative assay performance close to the LOQ (0.5 ng/mL), n=18.

Expected Concentration (ng/mL)	Calculated Concentration (ng/mL)	CV (%)	Accuracy (%)	Mean S/N
0.5	0.55 ± 0.07	13.3	110%	47:1
1	0.99 ± 0.10	10.5	99%	74:1
2	1.92 ± 0.30	15.8	96%	165:1
5	4.70 ± 0.83	17.6	94%	192:1
10	9.77 ± 1.29	13.2	98%	200:1

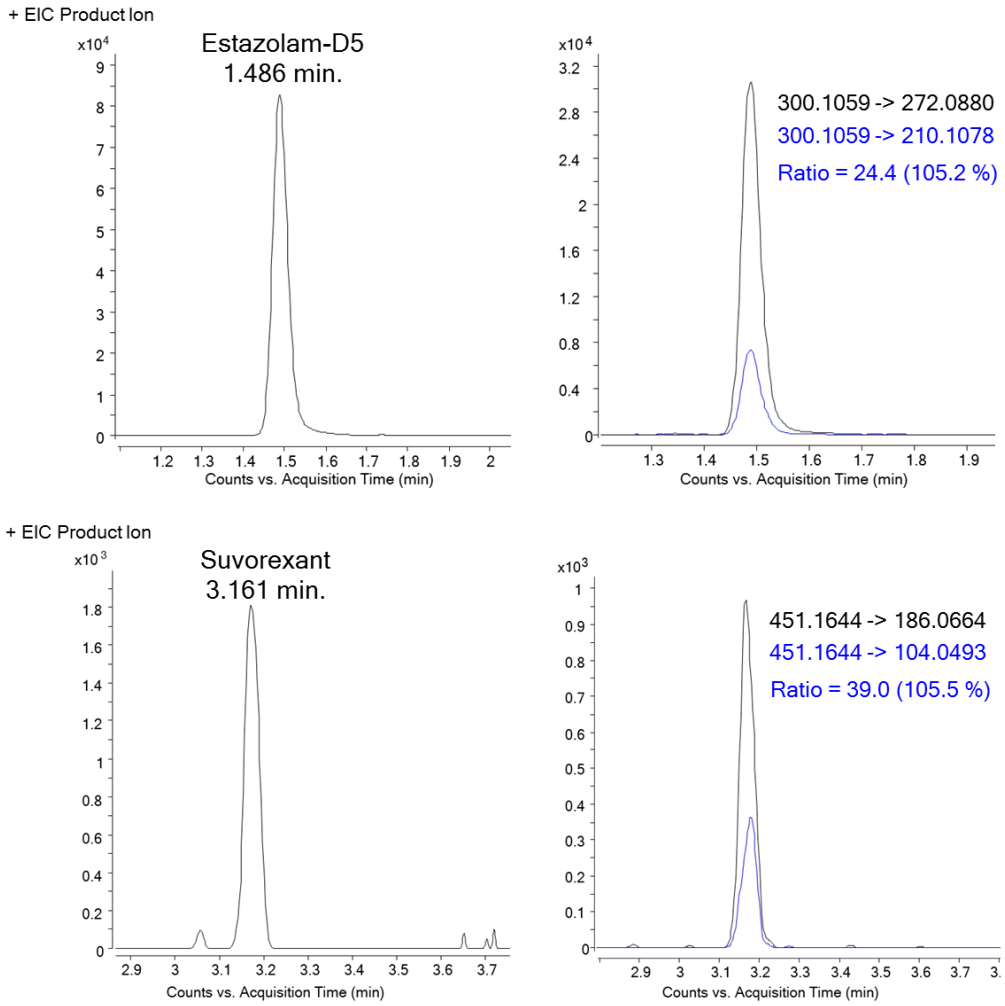


Figure 2.5. Extracted ion chromatograms for the internal standard at 100 ng/mL (upper) and suvorexant at the LOQ of 0.5 ng/mL (lower).

Coefficients of determination (R^2) were generated for linear, quadratic, weighted and non-weighted calibration models. The presence of heteroscedasticity (a change in variance across concentration levels) was evaluated using residual plot analysis. Systematic bias at high concentrations was observed for linear calibration models, regardless of weighting (**Figure 2.6**). A weighted quadratic model was selected due to improved performance at both the low and high end of the calibration. The average R^2 value using the 1/x quadratic model was 0.995 (n=5).

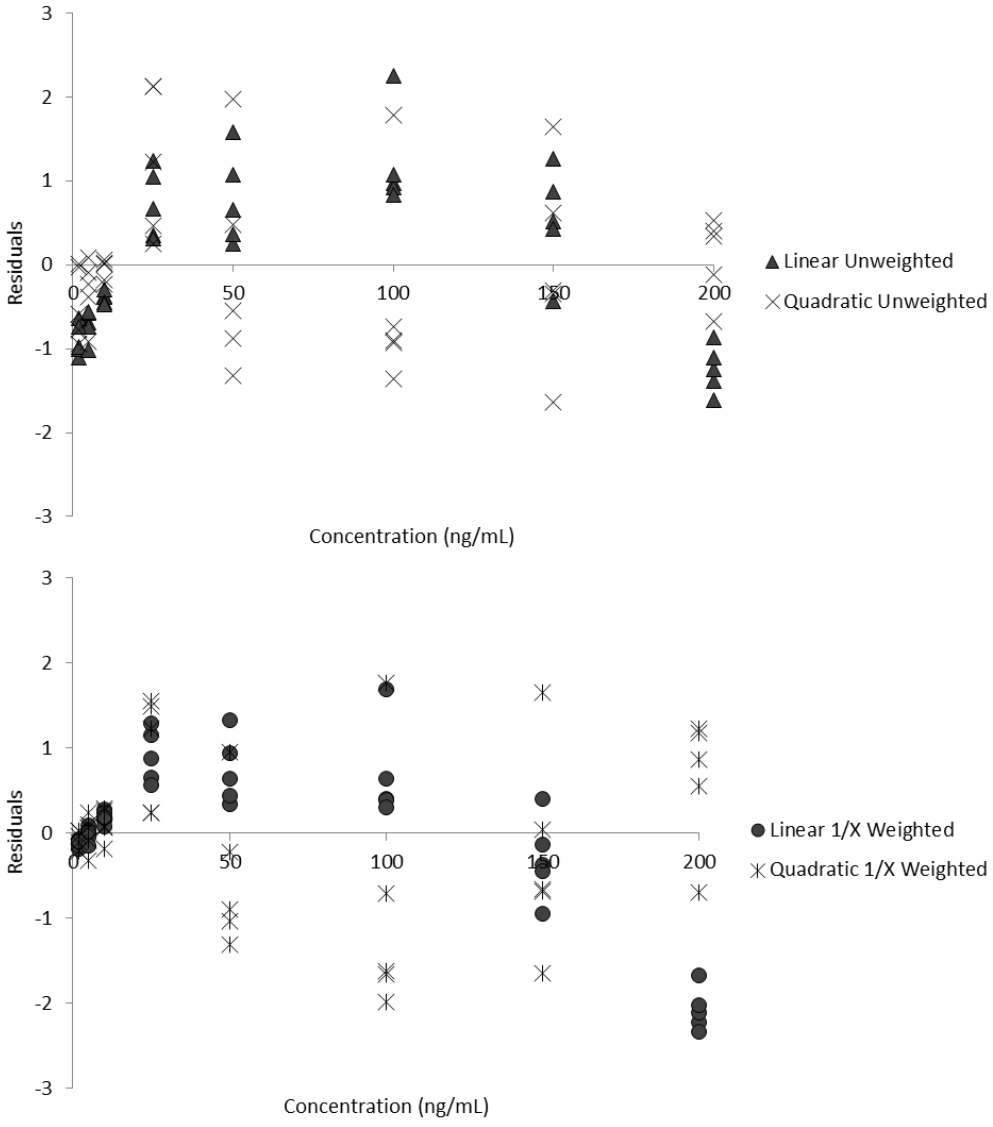


Figure 2.6. Evaluation of calibration models using residual plot analysis. Unweighted (upper) and weighted (lower).

Precision and accuracy were assessed by fortification of pooled blood with suvorexant at low, medium, and high concentrations (5, 50, and 160 ng/mL) in triplicate over five days. Intra-assay precision (n=5) was 10%, 4%, and 5% for low, medium, and high concentrations, respectively. Inter-assay precision (n=15) was 13%, 5%, and 8% for each concentration, respectively. Accuracy ranged from 95 to 102% for all concentrations.

Values fell within the 20% acceptance criteria for intra-assay precision, inter-assay precision, and bias (80–120% accuracy) (**Table 2.2**).

Table 2.2. Accuracy and precision at low (5 ng/mL), medium (50 ng/mL), and high (160 ng/mL) concentrations.

Concentration (ng/mL)	Intra-assay CV (%) n=5	Inter-assay CV (%) n=15	Accuracy (%) n=15
5	10%	13%	102%
50	4%	5%	99%
160	5%	8%	95%

No carryover was observed with the injection of blank matrix following the highest suvorexant calibrator (200 ng/mL) (n=3). Processed samples were stable in the autosampler compartment for 24 h post-extraction. Accuracy at 0, 6, 18 and 24 h was 94, 95, 84 and 82% respectively, and precision was 0.1–2.7% (n=3). However, by 48 h quantitative accuracy was significantly diminished (66%).

Ion suppression/enhancement was assessed qualitatively using post-column infusion. No qualitative interferences were observed with the injection of ten drug-free blood samples and syringe infusion of suvorexant and internal standard (**Figure 2.7**). In addition, matrix effects were determined quantitatively with post-extraction addition using a low and high concentration (20 ng/mL and 100 ng/mL). Percent matrix effects were calculated at each concentration by comparing analyte peaks of neat standards to matrix samples fortified after extraction. The mean matrix effect at 20 ng/mL was 16% with a CV of 9% (n=20). At 100 ng/mL the mean matrix effect was 15% with a CV of 8% (n=20).

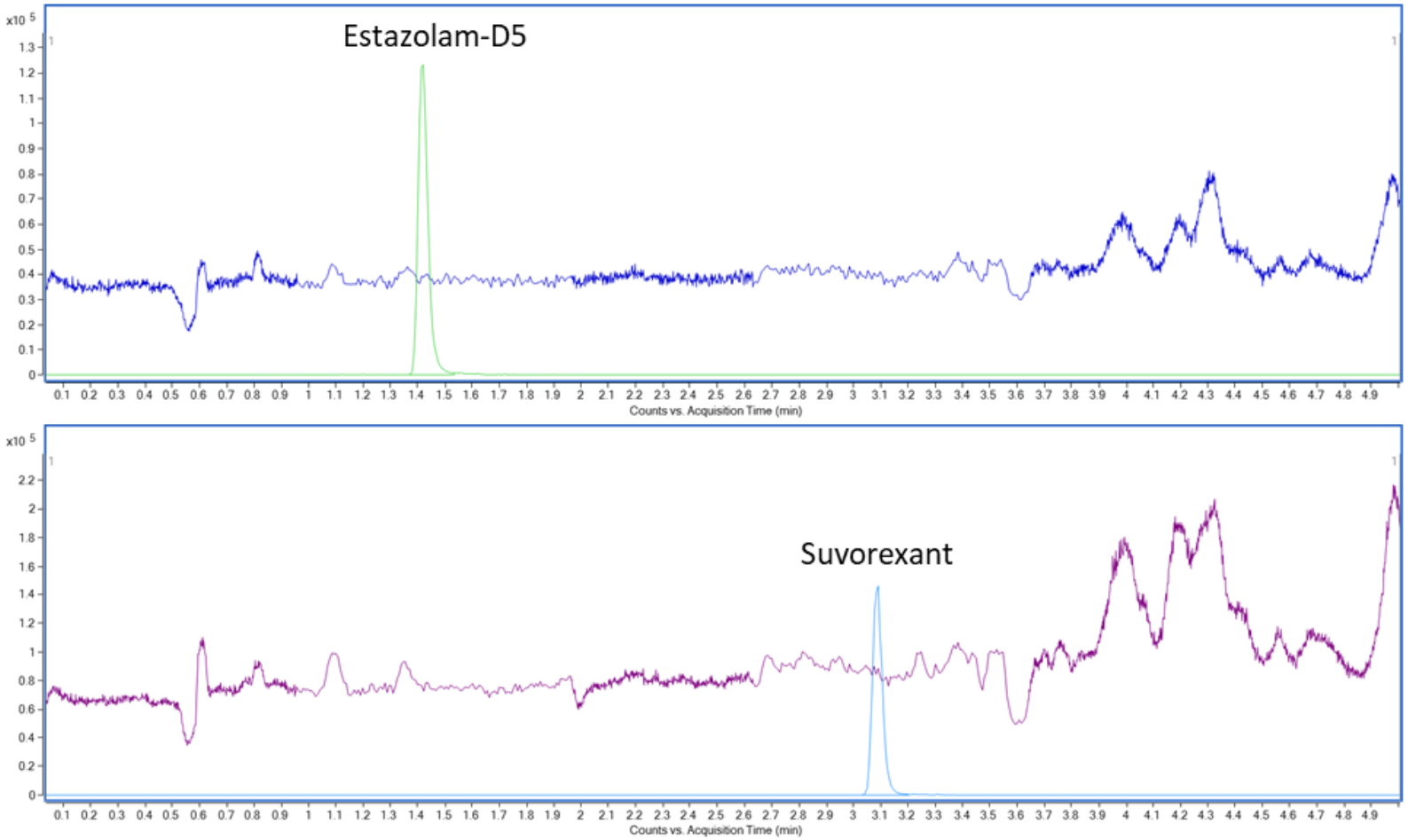


Figure 2.7. Evaluation of ion suppression on the internal standard using post-column infusion.

Current guidelines require drug interferences to be evaluated qualitatively, not quantitatively (13). Drug interferences were evaluated using negative and positive blood controls containing suvorexant (0, 10, and 100 ng/mL) and IS (100 ng/mL) in the presence of the fifty-three common drugs listed previously (potential interferents) at a tenfold and 100-fold higher concentration (1000 ng/mL). Interferences were evaluated qualitatively and quantitatively. Although no qualitative interferences were observed, a quantitative interference was identified. Further investigation revealed that the coelution of sertraline with the internal standard was responsible **(Figure 2.8)**. Although the coelution did not influence retention time or ion ratios used for qualitative purposes, the decreased ionization efficiency in the source (in the presence of excess interferent), resulted in significant bias. The hypothesis that this quantitative interference was due to "competition" between the IS and sertraline to ionize was further investigated by evaluating the magnitude of the bias using different proportions of interferent and drug.

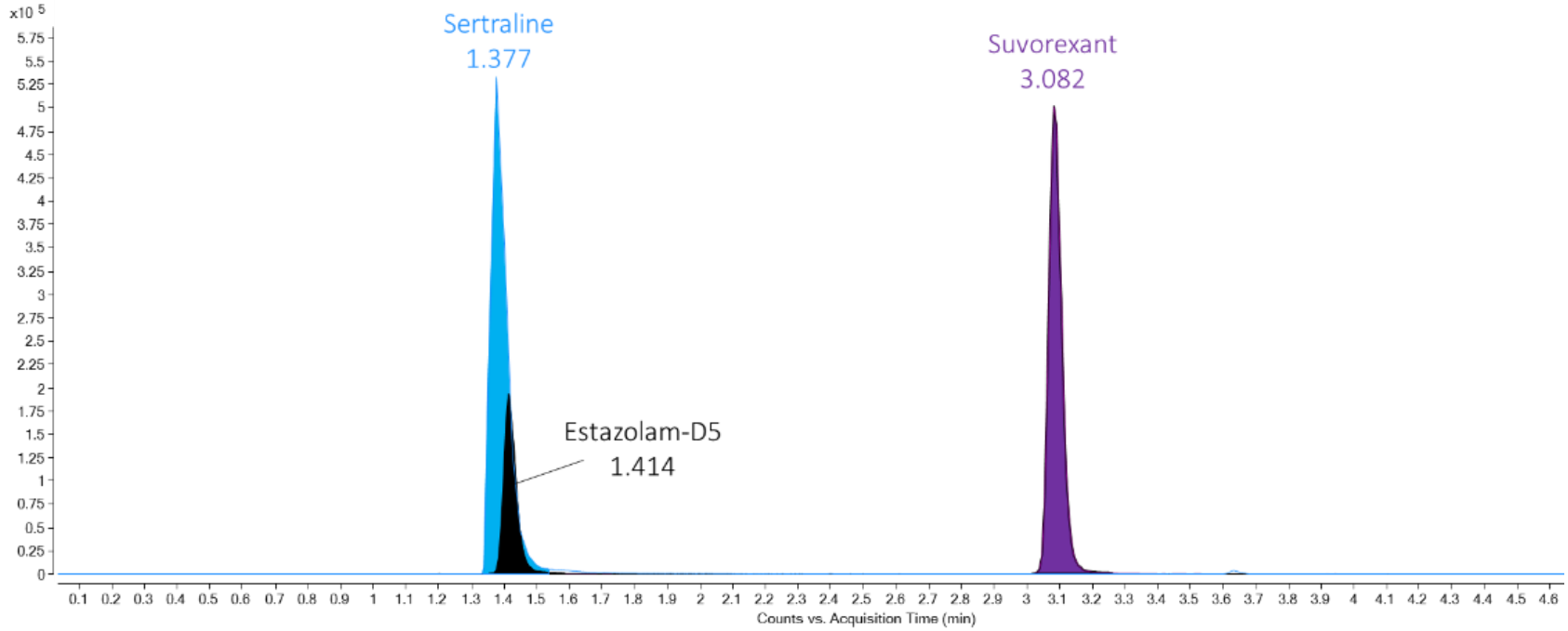


Figure 2.8. Chromatographic separation of sertraline, estazolam-D5 (IS) and suvorexant.

Sertraline interference was quantitatively evaluated in triplicate with concentrations ten-fold lower (1:10), equivalent (1:1), and ten-fold higher (10:1) concentration, relative to the internal standard (100 ng/mL). Quantitative concentrations were within the expected range when the interferent (sertraline) to IS concentration ratios were 1:10 and 1:1. Bias using triplicate measurements were −0.2 ± 11.3% and 8.8 ± 5.6%, respectively. However, a significant bias (82.5 ± 11.9%) was observed when sertraline was present at 10-fold higher concentration than the IS (**Figure 2.9**). These results highlight the potential for coeluting species to decrease overall ionization efficiency due to capacity-limited source ionization. This phenomenon, particularly in fast LC analysis where multiple drugs are simultaneously quantified, deserves additional attention. An excess of any coeluting drug at a much higher concentration than the target analyte has the potential to produce this effect during electrospray ionization. These effects can be somewhat mitigated by avoiding fast LC methods with excessively short run times, using minimal specimen, injection volume, and selective sample clean-up steps. If commercially available, the use of a deuterated suvorexant standard would eliminate this issue. These results highlight the need to critically evaluate interferences both qualitatively and quantitatively, particularly if isotopically labeled internal standards are not used.

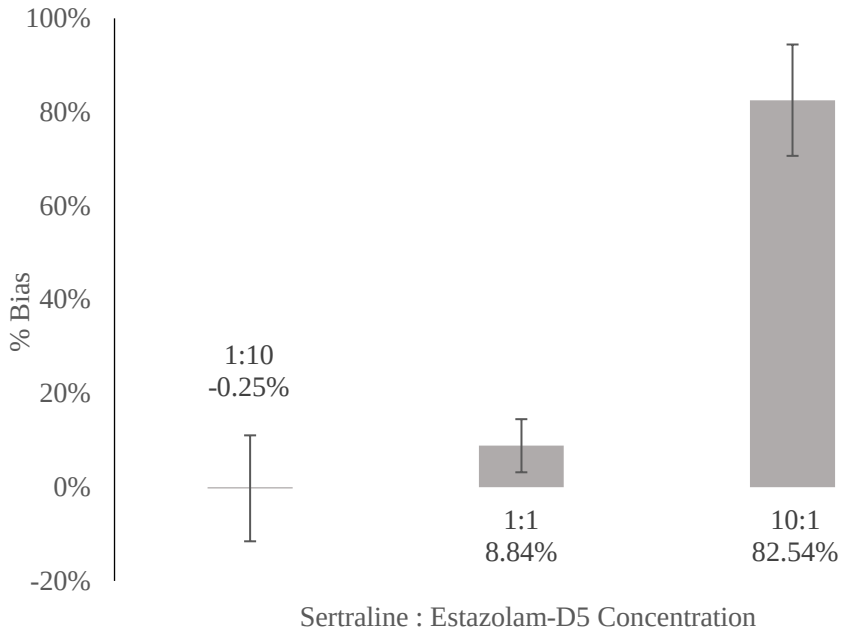


Figure 2.9. Potential for systematic bias in electrospray ionization due to coelution. Bias was evaluated using sertraline:estazolam-D5 concentration ratios of 1:10, 1:1 and 10:1. Data represents the mean ± 1 SD (n=3).

Currently, laboratories are not required to evaluate quantitative interferences (13) but this is highly encouraged in LC/MS-based assays. Systematic bias caused by reduced ionization efficiency in the electrospray source is not evident from peak shape, ion ratios or retention times, all of which may be within acceptable ranges.

Conclusion

Sedative hypnotic drugs feature prominently in forensic toxicology investigations, but to date there have been no published reports that describe the analysis of suvorexant in whole blood using LC-Q/TOF-MS. Forensic toxicology laboratories must have methods of analysis available in order to determine its role in human performance and death investigation casework. A new analytical procedure is described for the quantification of

suvorexant using LC-Q/TOF-MS. A limit of detection of 0.5 ng/mL was achieved using only 0.5 mL whole blood. This technique improves upon previously published techniques in urine that were capable of detecting suvorexant in urine at 10 ng/mL and 5 ng/mL using gas chromatography–mass spectrometry and LC-Q/TOF-MS, respectively. Furthermore, this new technique can be readily adapted to existing acidic/neutral liquid-liquid extraction protocols that are already in widespread forensic use.

References

1. Cox, C.D., Breslin, M.J., Whitman, D.B., Schreier, J.D., McGaughey, G.B., Bogusky, M.J., et al. (2010) Discovery of the dual orexin receptor antagonist [(7R)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl]methanone (MK-4305) for the treatment of insomnia. *Journal of Medicinal Chemistry*. **53**, 5320-5332.
2. Breidinger, S.A., Simpson, R.C., Mangin, E. and Woolf, E.J. (2015) Determination of suvorexant in human plasma using 96-well liquid–liquid extraction and HPLC with tandem mass spectrometric detection. *Journal of Chromatography B*. **1002**, 254-259.
3. Yang, L. (2014). Suvorexant: first global approval. *Drugs*, **74**, 1817-1822.
4. U.S. Food and Drug Administration (2013) Suvorexant Tablets Insomnia Indication, Peripheral & Central Nervous System Drugs Advisory Committee Meeting, NDA 204569. Available from: https://wayback.archiveit.org/7993/20170405225048/https://www.fda.gov/downloads/AdvisoryCommittee/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAvisoryCommittee/UCM352970.pdf (accessed November 17, 2018).
5. U.S. Drug Enforcement Administration (2014) Schedules of Controlled Substances: Placement of Suvorexant into Schedule IV. Final Rule. *Federal Register*. **79**, 51243-51247.
6. Uemura, N., McCrea, J., Sun, H., Donikyan, M., Zammit, G., Liu, R., et al. (2015) Effects of the orexin receptor antagonist suvorexant on respiration

during sleep in healthy subjects. *The Journal of Clinical Pharmacology*, **55**, 1093-1100.

7. Bennett, T., Bray, D. and Neville, M.W. (2014) Suvorexant, a dual orexin receptor antagonist for the management of insomnia. *P & T: A Peer-Reviewed Journal for Formulary Management*. **39**, 264-266.
8. Ciu, D., Cabalu, T., Lai Yee, K., Small, J., Li, X., Liu, B., et al. (2016) In vitro and in vivo characterisation of the metabolism and disposition of suvorexant in humans. *Xenobiotica*. **46**, 1-14.
9. Citrome, L. (2014) Suvorexant for insomnia: a systematic review of the efficacy and safety profile for this newly approved hypnotic – what is the number needed to treat, number needed to harm and likelihood to be helped or harmed? *International Journal of Clinical Practice*. **68**, 1429-1441.
10. Patel, K.V., Aspesi, A.V., Evoy, K.E. (2015) Suvorexant: a dual orexin receptor antagonist for the treatment of sleep onset and sleep maintenance insomnia. *Annals of Pharmacotherapy*. **49**, 477-483.
11. Waters, B., Hara, K., Ikematsu, N., Takayama, M., Matsusue, A., Kashiwagi, M., et al. (2018) Tissue distribution of suvorexant in three forensic autopsy cases. *Journal of Analytical Toxicology*. **42**, 276-283.
12. Sullinger, S., Bryand, K. and Kerrigan, S. (2017) Identification of suvorexant in urine using liquid chromatography-quadrupole/time-of-flight mass spectrometry (LC-Q/TOF-MS). *Journal of Analytical Toxicology*. **41**, 224-229.
13. Scientific Working Group for Forensic Toxicology (2013). Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method

Validation in Forensic Toxicology. *Journal of Analytical Toxicology*. **37**, 452-474.

14. (2002) Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, *Official Journal of the European Communities*, **L 221**, 8-36.
15. Iqbal, M., Ezzeldin, E., Khalil, N.Y., Al-Rashood, S.T.A. and Al-Rashood, K.A. (2017) Simple and highly sensitive UPLC-ESI-MS/MS assay for rapid determination of suvorexant in plasma. *Journal of Analytical Toxicology*. **41**, 114-120.
16. Carson, M. and Kerrigan, S. (2017) Quantification of suvorexant in urine using gas chromatography/mass spectrometry. *Journal of Chromatography B.* **1040**, 289-294.

CHAPTER III

IDENTIFICATION OF SUVOREXANT IN BLOOD USING LC-MS/MS: IMPORTANT CONSIDERATIONS FOR MATRIX EFFECTS AND QUANTITATIVE INTERFERENCES IN TARGETED ASSAYS[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology.*

[1] Skillman, B., and Kerrigan, S. (2019) Journal of Analytical Toxicology. https://doi.org/10.1093/jat/bkz083. Reprinted with permission of the publisher.

Abstract

Suvorexant (Belsomra®) is a novel dual orexin receptor antagonist used for the treatment of insomnia. The prevalence of suvorexant in forensic samples is relatively unknown, which demonstrates the need for robust analytical assays for the detection of this sedative hypnotic in forensic toxicology laboratories. In this study, suvorexant was isolated from whole blood using a simple acidic/neutral liquid-liquid extraction followed by analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). Matrix effects were evaluated qualitatively and quantitatively using various extraction solvents, proprietary lipid clean-up devices, and source conditions. The method was validated in terms of limit of detection, limit of quantitation, precision, bias, calibration model, carryover, matrix effects, and drug interferences. Electrospray is a competitive ionization process whereby compounds in the droplet compete for a limited number of charged sites at the surface. As such, it is capacity-limited, and LC-MS based techniques must be carefully evaluated to ensure that matrix effects or coeluting drugs do not impact quantitative assay performance. In this report, we describe efforts to ameliorate such effects in the absence of an isotopically labeled internal standard. Matrix effects are highly variable and heavily dependent on the physico-chemical properties of the substance. Although there is no universal solution to their resolution, conditions at the electrospray interface can mitigate these issues. Using this approach, the LC-MS/MS assay was fully validated and limits of detection and quantitation of 0.1 and 0.5 ng/mL suvorexant were achieved in blood.

Keywords: Suvorexant, Matrix effects, Ion suppression, LC-MS/MS, LC-Q/TOF-MS

IDENTIFICATION OF SUVOREXANT IN BLOOD USING LC-MS/MS: IMPORTANT CONSIDERATIONS FOR MATRIX EFFECTS AND QUANTITATIVE INTERFERENCES IN TARGETED ASSAYS

Introduction

Suvorexant (MK-4305) is among a novel class of medications called Dual Orexin Receptor Antagonists (DORAs) that are used for the treatment of insomnia (1). According to the National Institutes of Health, insomnia affects approximately one-third of the American population and ten percent of these individuals experience next-day impairment associated with insomnia (2). Suvorexant is marketed under the tradename Belsomra® by Merck and Co., Inc. and it is thought to have a lower abuse potential than other traditional sedative hypnotics, which could make it a popular option for newly-prescribed insomnia patients (3,4). The Food and Drug Administration (FDA) approved suvorexant in August 2014 and it was placed under Schedule IV of the Controlled Substances Act before becoming commercially available in 2015 (3,5). Unlike conventional sedative hypnotics, suvorexant has no effect on gamma-aminobutyric acid (GABA) receptors, and instead inhibits orexin A and B in the lateral hypothalamus to control the transition from wakefulness to sleep (6). Suvorexant has a half-life of approximately 12 hours and peak plasma concentrations are typically reached within two hours of administration of a 10-mg oral dose, with bioavailability (~82%) decreasing as the dose increases (7,8). The volume of distribution (V_d) of suvorexant is 0.5-0.9 L/kg (9). Blood/plasma ratios have not been fully investigated and limited literature is available concerning the distribution of suvorexant in biological specimens of forensic interest (10). However, due to its relatively

long half-life and the prominence of sedative hypnotic medications in driving impairment and drug-facilitated sexual assault investigations, suvorexant is a drug of forensic interest.

Relatively few reports have described the analysis of suvorexant in biological samples, therefore little is known of its role in human performance toxicology investigations. The physicochemical properties of the drug may pose a challenge in terms of detection using traditional screening methods, and few labs routinely target the compound. Suvorexant is a neutral drug with a high boiling point (669°C). As a consequence, it is a late-eluting compound using common gas chromatography-mass spectrometry (GC-MS) stationary phases (11). Carson et al. published a GC-MS method for the detection of suvorexant in urine using a DB-5MS column (30 m x 0.25 mm) with a reduced (0.1 µm) film thickness, which resulted in an elution time of 11.7 minutes. The more commonly used DB-5 column with a 0.25 µm film thickness resulted in an elution time of nearly thirty minutes. This could be problematic in terms of detection in routine screening, because the drug may elute after data acquisition has ended. The GC-MS method described above was successfully validated in accordance with published guidelines, but in the absence of a commercially-available deuterated suvorexant analog, estazolam-D5 was as the internal standard (**Figure 3.1**) (11,12). Although isotopically labeled internal standards are generally preferred, these compounds share the 7-membered azepane/azepine ring, a heterocyclic triazole, and a chlorine. Waters et al. also published a fast GC-MS screening method for suvorexant that resulted in a retention time of 5.25 minutes, which was achieved using tandem GC columns. However, the authors also analyzed suvorexant standard under commonly used conditions for GC-MS screening and

reported a retention time of 23.7 minutes, also acknowledging that the compound could be easily missed by traditional GC-MS identification (10).

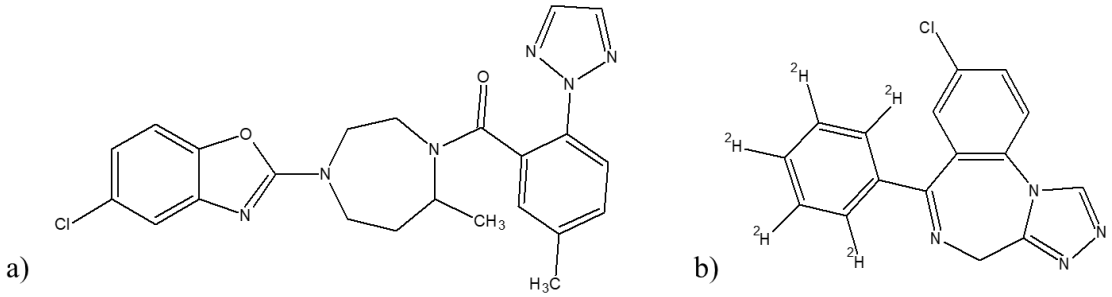


Figure 3.1. Chemical structures of suvorexant (a) and the internal standard, estazolam-D5 (b).

Liquid chromatography-mass spectrometry (LC-MS) techniques are some of the most versatile and increasingly-used analytical methods in forensic toxicology laboratories. LC-MS approaches have many advantages over traditional gas chromatography-mass spectrometry techniques, including the ability to analyze compounds with increased polarity, thermal lability and poor volatility. LC-MS can also allow for the detection of analytes that would otherwise require derivatization using GC-MS. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is commonly used to detect and quantify analytes of interest due to its high selectivity and sensitivity.

Due to its recent introduction to the market, there have been relatively few published reports that describe the analysis of suvorexant in biological samples using LC-MS-based methods. The drug manufacturer (Merck & Co.) described a method for the detection of suvorexant in plasma during a clinical study using LC-MS/MS. An isotopically-labeled suvorexant standard (suvorexant-$^{13}C^{2}H_3$) synthesized in-house was used for method development (13). Although the method demonstrated sensitivity over a

range of 1-1000 ng/mL, only one m/z transition was utilized for identification which is insufficient for forensic purposes (14).

Methods for the detection of suvorexant in plasma were also published by Iqbal et al. using LC-MS/MS (15,16). In a related study, they described the use of dispersive liquid-liquid micro-extraction and LC-MS/MS analysis to identify suvorexant in human and rat urine (17). Although the method resulted in an LOD of 0.1 ng/mL and an LOQ of 0.3 ng/mL, carbamazepine was used as the internal standard (IS) in the latter two reports. Not only does carbamazepine not bear any structural similarity to suvorexant, but it is also a commonly encountered anticonvulsant, making it a poor candidate for use as an internal standard. We previously reported methods to detect suvorexant in urine and blood using acidic/neutral liquid-liquid extraction (LLE) and liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS) (18,19). Both methods utilized estazolam-D5 as the internal standard and were fully validated for forensic use. Limits of detection and quantitation in blood were 0.5 ng/mL (19), with a corresponding LOD and LOQ of 0.5 and 5 ng/mL in urine (18).

The only other published report for suvorexant in blood used LC-MS/MS. Waters et al. identified suvorexant in three case specimens using fast-GC-MS screening and LC-MS/MS quantitation. The LC-MS/MS method was validated for blood and urine using diazepam-D5 as the internal standard. The LOD and LOQ for both blood and urine were 0.5 ng/mL and 1 ng/mL, respectively. The method was then applied to authentic blood, urine and homogenized tissues that had been subjected to protein precipitation followed by phospholipid removal using Agilent Captiva ND Lipid cartridges (10).

When using LC-MS based techniques, ion suppression and enhancement are important considerations, particularly when analyzing complex matrices (20). These can be assessed qualitatively using post-column infusion techniques, or quantitatively using post-extraction addition. Both approaches have merit. Post-extraction addition is a static technique that allows the matrix effect (ME) to be numerically estimated at the retention time of the analyte. In contrast, post-column infusion is a dynamic technique because it identifies chromatographic regions where an analyte could be susceptible to ME. Ideally, ion suppression or enhancement should not exceed 25% and should be reproducible between matrices (CV <15%) (12). Although both ion suppression and enhancement can occur, ion suppression is more common than enhancement, and electrospray ionization (ESI) is more susceptible to matrix effects than atmospheric pressure chemical ionization (APCI) (21,22). Of the published LC-MS methods for suvorexant, only the method developed by Merck used APCI (13). Although tandem (QQQ) and high resolution mass spectrometry (HRMS) techniques are considered to be highly selective, mass filtering occurs after separation has been achieved. Sources of ion suppression may include co-eluting compounds, metabolites, degradation products, endogenous compounds within the matrix itself, and exogenous compounds introduced during sample preparation (21,22). Matrix effects are also heavily dependent on the biological sample (i.e. urine, blood, plasma) and preparation techniques (extraction type). During quantitative analysis, matrix effects can lead to both positive and negative bias, depending on whether they coelute with the analyte or the internal standard. Removal of every potential matrix interferent is no more feasible than absolute chromatographic resolution of every possible compound. This demonstrates the importance of using stable isotope labeled internal standards (SIL-IS)

when available (20,22). These demonstrate near identical behavior to the analyte during sample preparation, separation and ionization, which can compensate for matrix effects (21). Standard addition techniques can also compensate for ion suppression. However, this method requires a much larger quantity of specimen due to multiple sampling, making it an unpopular choice for routine forensic analyses where specimen quantity may be limited.

Particularly for new or novel substances, a SIL-IS may not be commercially available. This further highlights the need to critically evaluate ion suppression and enhancement both qualitatively and quantitatively during method development. Any substance that coelutes with the compound of interest will compete with the analyte during ionization in the source. This can effectively decrease ionization for an analyte, particularly if there is a large excess of coeluting substance. The potential for reduced ionization efficiency due to matrix effects and other co-eluting drugs of interest may not be evident if interferences are evaluated only qualitatively. Although published standards only require interferences from other drugs to be evaluated qualitatively (12), the practice in our laboratory has been to evaluate quantitatively, as well as qualitatively. A large excess of a coeluting drug can suppress ionization in much the same way as matrix components. However, the decreased overall efficiency will not impact retention time or cause ion ratios to be out of range. As a consequence, ion suppression of the analyte in an "unknown" sample will not be discernable during analysis, potentially contributing to a negative bias in the quantitative result. Conversely, if the internal standard is suppressed, a positive bias during quantitative analysis is possible. Although careful monitoring of the absolute internal standard intensity between samples (calibrator, controls, samples) can help identify the latter, no such resolution is possible for compounds that coelute with the analyte in

unknown case specimens. In a previously validated method to identify suvorexant in blood using LC-Q/TOF-MS, no matrix effects were identified. However, a significant bias from sertraline (which coeluted with the internal standard) was identified quantitatively, but not qualitatively (19). In this report, we discus differences in assay performance in terms of ion suppression caused by matrix and other drugs, using both LC-Q/TOF-MS and LC-MS/MS.

Methods

Chemicals and Reagents

Suvorexant ([(7R)-4-(5-chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3,-triazol-2-yl)phenyl]methanone) was purchased from Cayman Chemical as a powder (Ann Arbor, MI). Estazolam-D5 internal standard (100 µg/mL) was purchased as a methanolic standard from Cerilliant Corp. (Round Rock, TX). Fifty-two common drugs were received as methanolic standards (1 mg/mL) from Cerilliant Corp. (Round Rock, TX), which included benzodiazepines, barbiturates, and sedative hypnotic (z-drugs), as listed in **Table 3.1**.

Table 3.1. Common drugs evaluated in the interference study.

Common Drugs		**Z-Drugs**
11-nor-9-carboxy-delta-9-THC	methaqualone	zaleplon
acetaminophen	morphine	zolpidem
amitriptyline	oxycodone	zopiclone
amphetamine	oxymorphone	
bupropion	phencyclidine	**Barbiturates**
caffeine	phenytoin	amobarbital
carbamazepine	propoxyphene	butalbital
carisoprodol	pseudoephedrine	pentobarbital
cocaine	salicylic acid	phenobarbital
codeine	sertraline	secobarbital
cyclobenzaprine	THC	
dextromethorphan	tramadol	**Benzodiazepines**
fluoxetine	trazodone	7-aminoclonazepam
gabapentin	valproic acid	7-aminoflunitrazepam
hydrocodone		alprazolam
hydromorphone		clonazepam
ketamine		diazepam
MDMA		flurazepam
meperidine		nordiazepam
meprobamate		oxazepam
methadone		temazepam

MDMA, methylenedioxymethamphetamine; THC, tetrahydrocannabinol.

Acetic acid (glacial), toluene (ACS grade) and sodium acetate (ACS grade) were obtained from Mallinckrodt Chemicals (St. Louis, MO). LC/MS grade methanol, LC/MS grade acetonitrile and ACS grade diethyl ether were obtained from J.T. Baker (Center Valley, MA). *N*-butyl chloride (99+%, pure), hexane (OptimaR), and ethyl acetate (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid (>95%) was obtained from Sigma-Aldrich (St. Louis, MO). An in-house Millipore Direct-Q ® UV Water Purification System (Billerica, MA) was used for the purification of deionized water.

Drug-free bovine blood containing 1% (w/v) sodium fluoride and 0.2% (w/v) potassium oxalate was purchased from Quad Five (Ryegate, MT). Sodium acetate buffer (0.4 M) at pH 3.6 was prepared for routine use in liquid-liquid extractions. Mobile phase

A consisted of 0.1% formic acid in deionized water and mobile phase B consisted of 0.1% formic acid in acetonitrile. Suvorexant stock solution was prepared at a concentration of 0.1 mg/mL in methanol. Working standards of suvorexant and estazolam-D5 were routinely prepared in methanol at a concentration of 1 µg/mL and 2 µg/mL, respectively.

LC Separation

LC conditions were identical for both LC-Q/TOF-MS and LC-MS/MS assays. A Poroshell EC-C18 column (2.1 x 100 mm, 2.7 µm particle size) and matching Poroshell 120 EC-C18 guard column (2.1 x 5 mm, 2.7 µm particle size) were maintained at 35°C. A flow rate of 0.4 mL/min was used with the following gradient elution profile: 40% B (0); 40-80% B (0-3 mins); hold 80% B (3-4 min); 80-40% B (4-5 mins). A post-equilibration time of 2 minutes was utilized before injection of the next sample, with a 6-second needle wash in between injections to prevent carryover.

Q/TOF-MS Analysis

LC-Q/TOF-MS analysis was performed using an Agilent Technologies 1290 Infinity Binary LC System coupled to a 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS system (Santa Clara, CA). Positive electrospray ionization (ESI) mode was used with the following manually optimized ionization conditions: 300°C gas temperature, 13 L/min gas flow, 45 psi (310 kPa) nebulizer pressure, 350°C sheath gas temperature, and 12 L/min sheath gas flow. The mass spectrometer was operated with a capillary voltage of 3000 V, nozzle voltage of 2000 V, and fragmentor voltage of 150 V. A collision energy of 50 eV was used for suvorexant with targeted MS/MS data acquisition. Two product ion transitions were used (m/z 451.1644 > <u>186.0664</u> and m/z 451.1633 > 104.0493). Quantitation ions are underlined. A collision energy of 30 eV for estazolam-D5 was used

with two ion transitions (m/z 300.1059 > 272.0875 and m/z 300.1059 > 210.1076) in targeted MS/MS mode. The MS scan rate was 5 spectra/second (100-1600 amu), isolation widths were 1.3 amu, mass tolerance was ± 5 ppm, and acquisition time was 200 (ms/spec) (19). Whole blood extracts were routinely reconstituted in a 1:1 mixture of mobile phase A/B (30 µL) and 2 µL was injected onto the LC-Q/TOF-MS for analysis.

MS/MS Analysis

LC-MS/MS analysis was performed using an Agilent 1290 Infinity Liquid Chromatograph System coupled to an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA). Positive ESI mode was used and initial ionization conditions were optimized using Agilent MassHunter Source Optimizer software as follows: 300°C gas temperature, 8 L/min gas flow, 20 psi (138 kPa) nebulizer pressure, 400°C sheath gas temperature, 10 L/min sheath gas flow, nozzle voltage of 0 V and a capillary voltage of 4000 V. For suvorexant and estazolam-D5, fragmentor voltages of 127 V and 140 V were used, respectively. Data was acquired in dynamic multiple reaction monitoring (dMRM) mode with two transitions for each species. The transitions monitored for suvorexant were m/z 451.2 > 186.0 (21 eV) and m/z 451.2 > 104.0 (73 eV). The transitions for estazolam-D5 were m/z 300.0> 272.1 at (24 eV) and m/z 300.0 > 210.1 at (48 eV). Dwell times for all transitions were 200 ms. Following observation of ion suppression using the software-optimized source conditions, the source conditions from the LC-Q/TOF-MS assay (described above) were evaluated on the LC-MS/MS. Whole blood extracts were routinely reconstituted in a 1:1 mixture of mobile phase A/B (30 µL) and 2 µL was injected onto the LC-MS/MS for analysis.

GC-MS Analysis

An Agilent 7890A Gas Chromatograph equipped with a 5975C Mass Selective Detector was used to identify suvorexant and potential matrix interferences that were observed during initial method development. Suvorexant was determined using the previously validated procedure (11). Chromatographic separation was achieved using a DB-5MS column (30 m x 0.25 mm) with a 0.1 µm film thickness. The inlet temperature was 280°C and the temperature gradient was as follows: 260°C for 0.1 min, ramp to 290°C at 30°C/min followed by 16 min hold (17.1 min total run time). A 10:1 split ratio was employed with a 2 µL sample injection volume. Six pre-injection and six post-injection methanol washes were performed between each sample injection. Electron impact (EI) ionization of 70 eV was used, and data was acquired using selected ion monitoring (SIM) acquisition following a 2.4 min solvent delay. Dwell times for estazolam-D5 (m/z 210.1, 264.1 and 299.1) and suvorexant (m/z 104.1, 186.1, and 450.2) were 50 ms and 75 ms, respectively.

Endogenous phospholipid screening was also performed using a generic full scan GC-MS method (40-550 Da) using a DB-5MS column (30 m x 0.25 mm) with a 0.1 µm film thickness. The inlet temperature was 250°C with the following temperature gradient: 160°C for 5 min with a ramp to 290°C at 30°C/min (for 9.167 min) for 18.5 min total run time. A 10:1 split ratio was employed with a 2 µL sample injection volume and twelve pre- and post- injection methanol washes.

Extraction

Suvorexant was isolated from whole blood using liquid-liquid extraction (LLE) as described earlier (19). Briefly, whole blood (0.5 mL) was fortified with IS to achieve a

final concentration of 100 ng/mL. Following the addition of 1 mL sodium acetate buffer (0.4 M pH 3.6) and *N*-butyl chloride (2.5 mL), samples were mixed on a rotary mixer (5 mins) and centrifuged (3000 rpm/ 1734 x g, 5 mins). Following removal of the supernatant, organic extracts were evaporated to dryness under nitrogen at 50°C and reconstituted in 30 µL methanol (for GC-MS) or 30 µL 1:1 mobile phase A/B (for LC-Q/TOF-MS and LC-MS/MS).

Unlike the previously validated LC-Q/TOF-MS method, significant ion suppression was observed using the LC-MS/MS assay. Due to the methods being identical (with the exception of source conditions and data acquisition) endogenous interferences were further explored. Initially, alternative extraction solvents were evaluated. Direct comparisons were made between *N*-butyl chloride, ether/toluene (1:1) and hexane/ethyl acetate (9:1) extraction solvents. Analyte recovery and endogenous matrix interferences were investigated. Additional phospholipid removal was assessed using three proprietary clean-up cartridges (1 mL) as follows: Agilent Captiva EMR- Lipid (Santa Clara, CA), Phenomenex Phree™ Phospholipid Removal Solutions (Torrance, CA), and Supelco Analytical HybridSPE™-Precipitation Technology (Sigma-Aldrich, St. Louis, MO).

Lipid removal was performed in accordance with the manufacturers' recommendations, using acetonitrile containing 1% formic acid as the precipitation agent. Since whole blood was used, samples were prepared for cartridge filtration by first performing offline protein precipitation. To 0.5 mL of blood, 1 mL cold acetonitrile (with and without 1% formic acid) was added with vortex mixing (n=2). Cold acetonitrile was used to enhance protein precipitation, and the performance of the extraction was evaluated with and without the addition of 1% formic acid. The samples were centrifuged at 3500

rpm (2360 x g) for 5 minutes and the supernatant (~1 mL) was decanted into commercial lipid removal cartridges. Samples were eluted under vacuum and the eluent was collected into glass conical tubes. Acetonitrile was evaporated under nitrogen (60°C) until near dryness and 1 mL of sodium acetate buffer (0.4 M, pH 3.6) was added, followed by LLE as described above.

Evaluation of Matrix Effects

Both post-column infusion and post-extraction addition are routinely used in our laboratory to investigate matrix effects during method development and validation. Elution of endogenous interferences during the chromatographic run was evaluated using ten drug-free blood extracts from independent sources. Suvorexant and internal standard were infused into the MS using a T-connector and a KDS 100 Legacy Single Syringe Pump (KD Scientific, Holliston, MA). Matrix effects were then evaluated quantitatively using post-extraction addition in which ten drug-free matrices were extracted in duplicate and fortified with low and high concentrations (20 and 100 ng/mL) of suvorexant and IS. Neat standards were prepared concurrently by fortifying mobile phase with equivalent amounts of drug. Matrix effects were determined by direct comparison of the ion abundance in the presence and absence of matrix. Average matrix effects should not exceed ±25% and the CV of the suppression or enhancement should not exceed 15%. Although no quantitative interferences were identified for the LC-Q/TOF-MS method, ion suppression exceeding 25% was identified at both concentrations using the initial LC-MS/MS assay. Source optimization for this assay was performed using MassHunter Source Optimizer software based on analytical response (intensity). Notably, these conditions differed slightly from the manually-optimized LC-Q/TOF-MS conditions developed previously.

In an effort to mitigate matrix effects, three extraction solvents and commercial lipid removal cartridges were evaluated as described above. Qualitative and quantitative matrix effects were directly compared using LC-MS/MS and LC-Q/TOF-MS assays, with and without additional lipid removal. Finally, the validated LC-Q/TOF-MS source parameters were applied to the LC-MS/MS method. Utilizing both sets of source conditions, matrix effects were directly compared using post-column infusion and post-extraction addition techniques.

Validation of LC-MS/MS and LC-Q/TOF-MS Assays

The LC-MS/MS method was validated in accordance with published recommendations (12) and performance was compared to the previously validated LC-Q/TOF-MS method (19). Parameters that were assessed included limit of detection, limit of quantification, precision, bias, calibration model, carryover, matrix effects and interferences.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined empirically by fortifying three independent sources of blood with suvorexant (0.1, 0.25, 0.5, 1, 2, 5, 10 ng/mL) and internal standard (100 ng/mL) in duplicate over three days. The LOD was defined as the lowest concentration of drug that produced a signal-to-noise (S/N) ratio greater than 3:1, ion ratios within 20% of the expected value, and a retention time within 2% of the known standard. The LOQ was defined as the lowest concentration of suvorexant to produce a S/N ratio greater than 10:1, ion ratios within 20% of the expected value, a retention time within 2% of the verified standard, and bias within 20%.

Precision and bias were evaluated using fortified pooled blood samples at identical concentrations as the previously validated LC-Q/TOF-MS method (5, 50, and 160 ng/mL

suvorexant and 100 ng/mL IS) in triplicate over five days. The precision and bias for each instrument were compared to evaluate performance of the assay at each concentration.

Calibration model was assessed using eight fortified whole blood calibrators (2, 5, 10, 25, 50, 100, 150, and 200 ng/mL suvorexant) over five days. Linear and quadratic models (with and without weighting) were evaluated using residual plot analysis and coefficients of determination. Carryover was studied by analyzing a drug-free extract immediately following the injection of the highest calibrator. The assay was determined to be carryover-free if no drug was detected (i.e. did not meet reporting criteria) when performed in triplicate.

Interferences from other commonly encountered drugs were analyzed by fortification of blood with more than 50 common drugs (**Table 3.1**). Positive and negative controls were evaluated using a 100-fold excess of interferent to analyte (i.e. 10 ng/mL suvorexant; 1000 ng/mL other drug). Interferences were assessed qualitatively (i.e. peak shape, retention time, chromatographic quality) and quantitatively (% bias). Upon isolation of potential drug interferences, controls were again analyzed using concentration ratios of 1:10, 1:1, and 10:1 interferent:analyte to determine the magnitude of quantitative biases resulting from any drug interference.

Results

Matrix Effects Using LC-Q/TOF-MS and LC-MS/MS

Matrix effects were evaluated qualitatively and quantitatively using the same drug-free matrices (n=10), all of which were obtained from independent sources of bovine blood. **Table 3.2** highlights the differences observed. Although matrix effects were well within acceptable criteria using the LC-Q/TOF-MS assay, the LC-MS/MS method exceeded

acceptability criteria at both low and high concentrations (20 and 100 ng/mL) for suvorexant (**Table 3.2**). Performance criteria were exceeded in terms of the magnitude of the suppression (>25%), and the variability between matrices (CV>15%). The LC-Q/TOF-MS assay performance was superior in this respect.

Table 3.2. Comparison of matrix effects using LC-MS/MS and LC-Q/TOF Assays. Parameters outside of acceptable limits are shown in bold.

	Average Matrix Effect (%CV, n=10)					
	LC-Q/TOF-MS		**LC-MS/MS (Initial)**		**LC-MS/MS (Final)**	
	Suvorexant	IS	Suvorexant	IS	Suvorexant	IS
Low (20 ng/mL)	16 (9%)	19 (7%)	**-35 (23 %)**	12 (5 %)	-8 (15%)	-5 (13%)
High (100 ng/mL)	15 (8%)	11 (9%)	**-26** (13 %)	6 (7 %)	-9 (6%)	10% (3%)

Assessment of matrix effects using post-column infusion further illustrates the issue in the LC-MS/MS assay. **Figure 3.2** depicts the change in absolute signal for both suvorexant and the internal standard during the five minute chromatographic run. No suppression or enhancement was observed for estazolam-D5 (1.4 min), but ion intensities for suvorexant (3.1 min) were clearly influenced by matrix components. Changes in signal intensity are expected when small, polar molecules (that are poorly retained) elute from the column. However, the suppression observed in the latter part of the chromatogram may be attributed to nonpolar or lipophilic compounds (e.g. phospholipids, cholesterol, fatty acids, fatty acid esters).

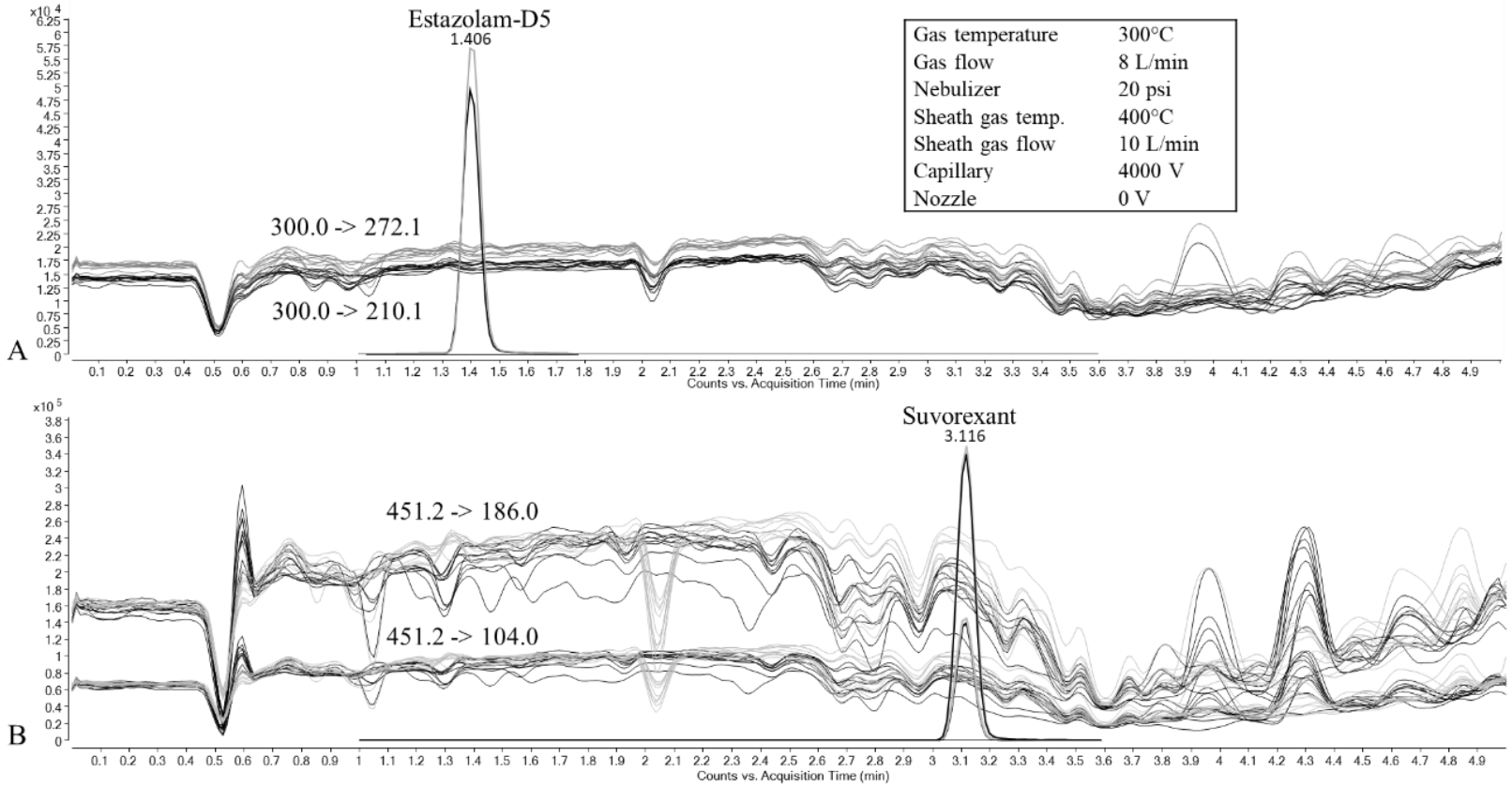


Figure 3.2. Initial LC-MS/MS matrix effects using post-column infusion (100 ng/mL). Overlaid extracted ion chromatograms for suvorexant and estazolam-D5 are shown for comparison.

This was further investigated using the previously validated GC-MS assay in combination with full-scan data acquisition (11). The persistence of cholesterol, fatty acids and fatty acid esters in the optimized LLE using *N-butyl* chloride is shown in **Figure 3.3**. These endogenous co-extractive species were identified only tentatively using MS library searching (NIST MS library).

Three solvent systems (*N*-butyl chloride, ether/toluene (1:1) and hexane/ethyl acetate (9:1)) were evaluated during the original LLE method development using LC-Q/TOF-MS. No significant differences were observed in terms of the analytical recovery of suvorexant between the solvents (19). These were re-evaluated using the LC-MS/MS assay from the standpoint of matrix effect. No qualitative or quantitative differences were observed between the solvents, and the same lipids persisted. Various commercial lipid clean-up devices were then investigated in an attempt to mitigate suppression. The devices were used as a pre-extraction clean-up technique by first performing protein precipitation on the fortified whole blood and subjecting the resulting supernatant to treatment in accordance with the manufacturers' recommendations. The presence of formic acid in the acetonitrile precipitation solvent resulted in negligible differences in extraction efficiency following the lipid clean-up, therefore cold acetonitrile without formic acid was used as the optimal protein precipitation reagent. The GC-MS screening method described above was used to evaluate the effectiveness of the lipid removal devices. Although several of the fatty acids and fatty acid esters remained, cholesterol was completely eliminated using the Agilent Captiva-EMR and Phenomenex Phree™ devices. Cholesterol still remained following the Supelco Phospholipid-Ultra cartridges (**Figure 3.4**). Due to the higher oven temperature used in the validated suvorexant assay, only cholesterol was present in the

targeted SIM method. However, its presence was not problematic because it was clearly resolved from suvorexant and the internal standard. **Figure 3.5** depicts the total ion chromatogram using the targeted SIM method following LLE, with and without additional phospholipid removal. Many of the proprietary lipid removal devices trap lipids based upon a combination of size exclusion and hydrophobic interactions. The latter may explain the decrease in suvorexant intensity, due to the high lipophilicity of the drug.

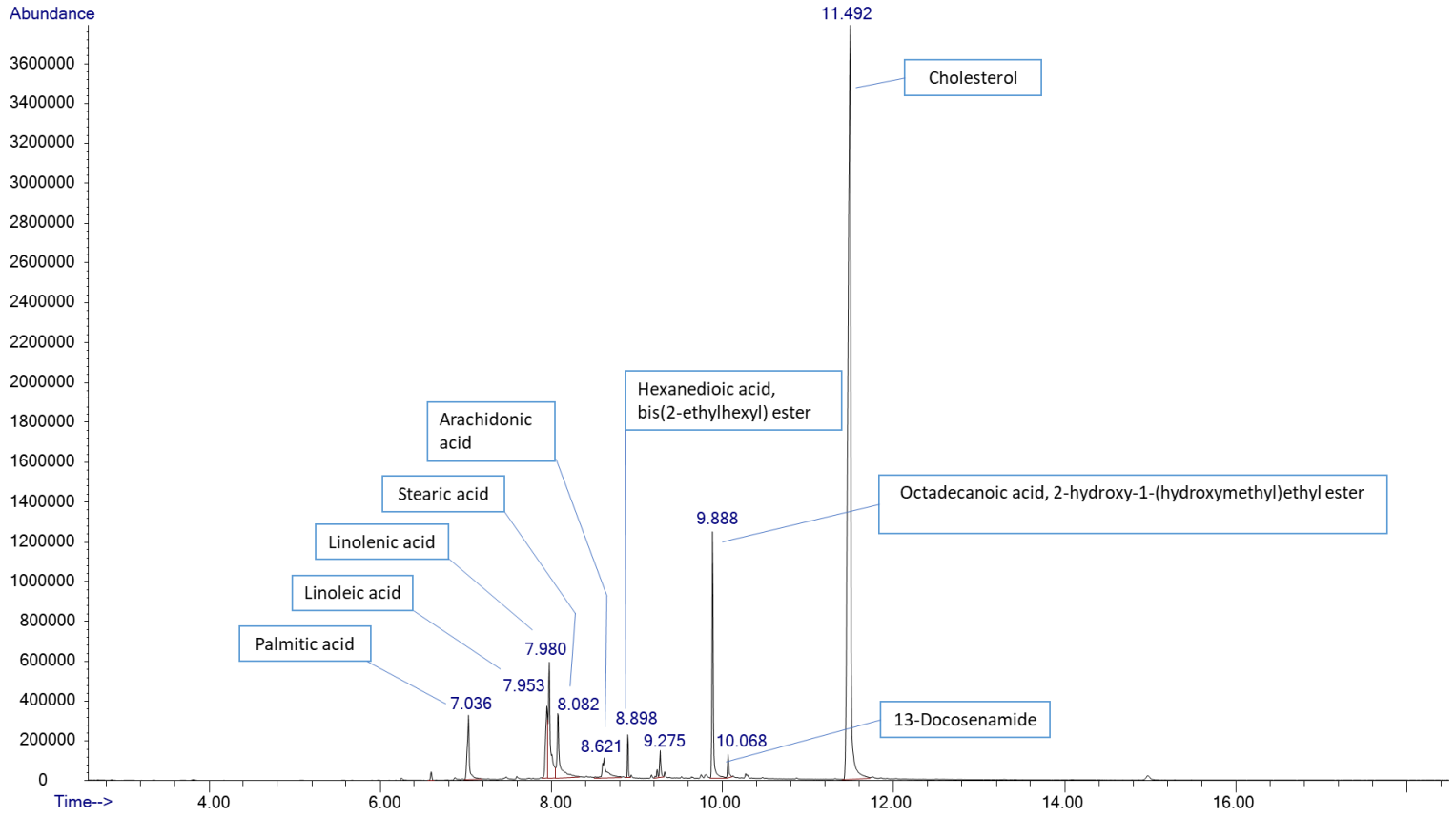


Figure 3.3. Total ion chromatogram following full scan GC-MS analysis of the liquid-liquid extract.

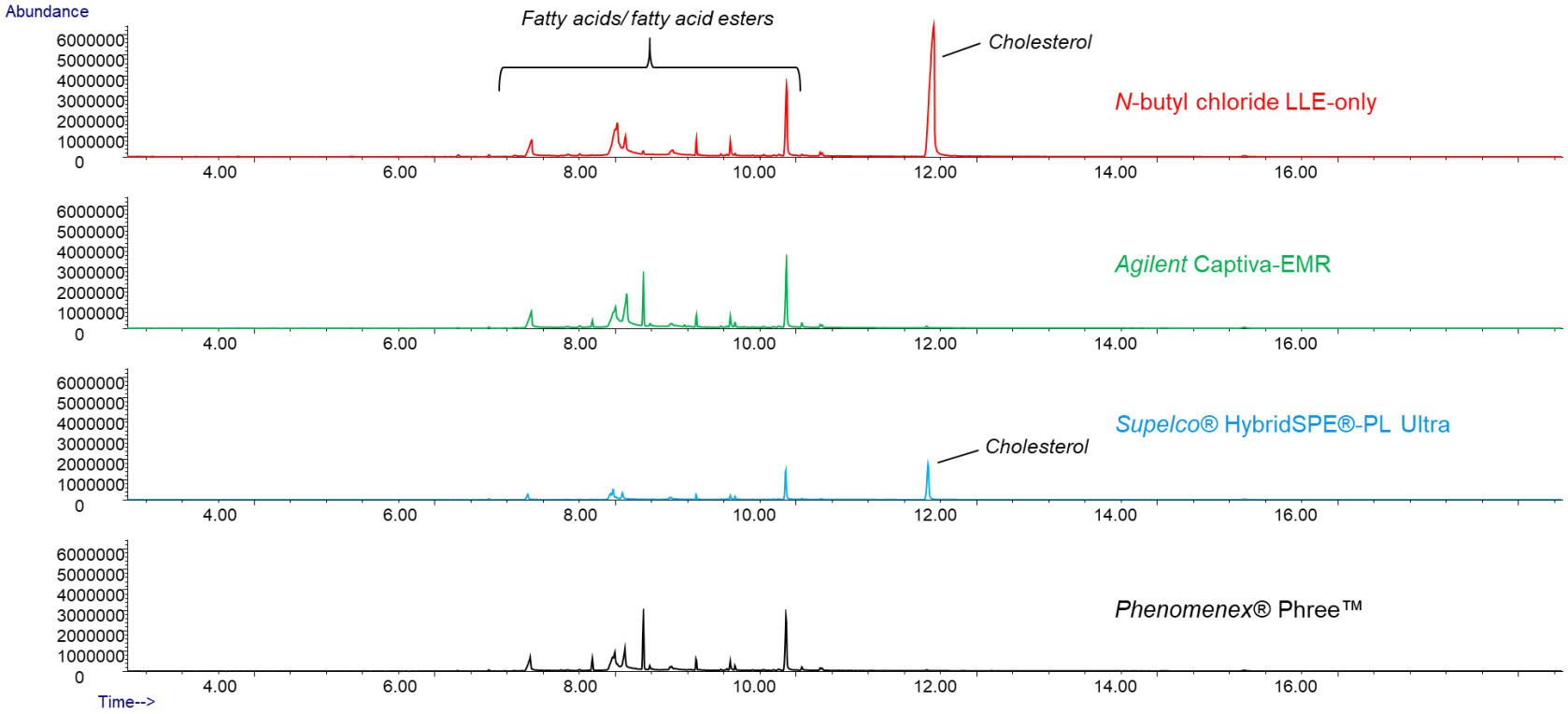


Figure 3.4. Persistence of fatty acids, fatty acid esters and cholesterol following cartridge-based lipid removal devices followed by LLE.

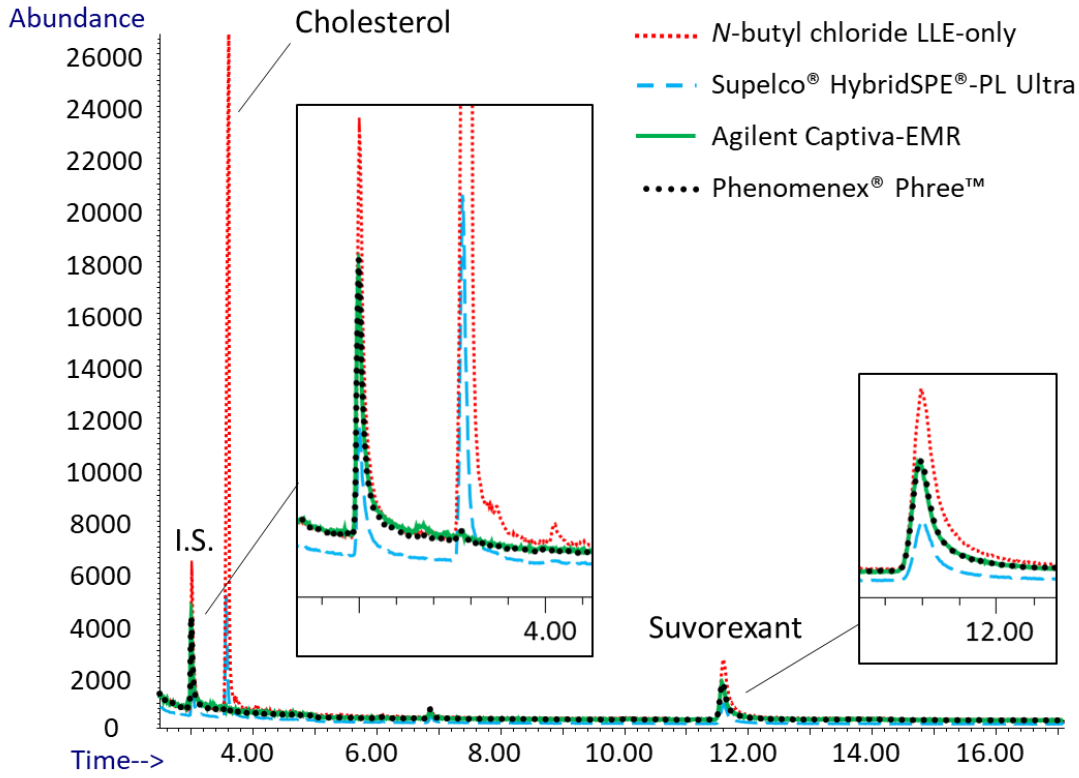


Figure 3.5. Total ion chromatogram following LLE, with and without additional phospholipid removal using the targeted GC-MS assay.

Based on their earlier performance, Agilent Captiva EMR-Lipid cartridges were further investigated. Mitigation of ion suppression was evaluated using post-column infusion. The same ten drug-free matrices with and without pre-extraction clean-up prior to LLE were injected while suvorexant and estazolam-D5 were constantly infused to the source. These samples were also analyzed by LC-Q/TOF-MS in parallel to determine if the additional lipid removal had any effect on the previously published results. The absence of matrix effects was confirmed using the LC-Q/TOF-MS assay (**Figure 3.6 A**). No suppression was present in LLE extracts and pre-extraction lipid removal was not necessary. In contrast however, significant suppression was present in the LC-MS/MS

assay and the pre-extraction lipid removal prior to LLE did not significantly improve matrix effects (**Figure 3.6 B**).

Although identical LC separation profiles were used, and both instruments were operated using positive ESI, the LC-Q/TOF-MS source parameters were manually optimized during method development. In contrast, the LC-MS/MS source parameters were optimized using Agilent MassHunter Source Optimization software in which the gas flows, voltages, and temperatures were selected to maximize the absolute response of the analyte. Unexpectedly, this resulted in unacceptable matrix effects using the LC-MS/MS assay.

Since the observed ion suppression was not resolved with solvent selection or additional pre-extraction phospholipid removal, source conditions were re-evaluated. When the source conditions for the published LC-Q/TOF-MS method were employed using the LC-MS/MS method, matrix effects were completely eliminated (**Figure 3.6 C**). Using this approach, the lipid removal step was not necessary. Modification of the source conditions reduced matrix effects for suvorexant to -8% and -9%, from -35% and -26% for low and high concentrations, respectively (**Table 3.2**). Although the signal intensity for suvorexant was slightly reduced, matrix interferences were completely mitigated. The manually optimized source parameters (**Table 3.3**) were used for the final validation of the LC-MS/MS assay, without the need for any additional sample preparation steps.

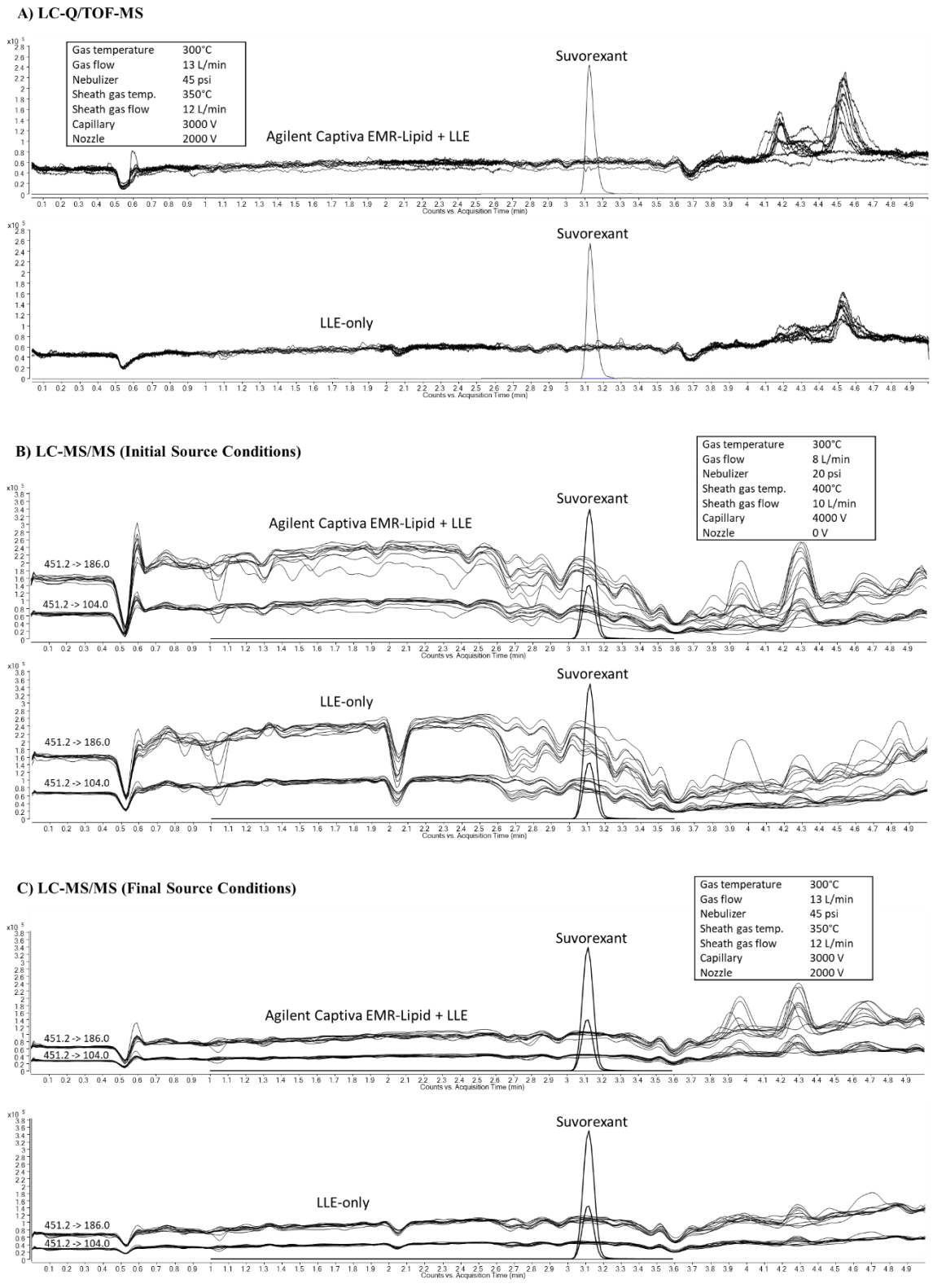


Figure 3.6. Comparison of LC-Q/TOF-MS and LC-MS/MS matrix effects using post-column infusion, with and without lipid removal.

Table 3.3. Initial and finalized source conditions used for the LC-MS/MS method validation.

Parameter	Initial	Final
Gas Temperature (°C)	300	300
Gas Flow (L/min)	8	13
Nebulizer (psi)	20	45
Sheath Gas Temp (°C)	400	350
Sheath Gas Flow (L/min)	10	12
Capillary (V)	4000	3000
Nozzle (V)	0	2000

Interferences from Other Drugs

Potential interferences from other drugs were investigated using the fifty-two common drugs listed previously (**Table 3.1**). Positive controls (10 ng/mL suvorexant, 100 ng/mL IS) and negative controls with internal standard only (100 ng/mL) were prepared with the addition of potential interferents at 100-fold excess concentrations (1000 ng/mL). No qualitative interferences were present for either suvorexant or the IS. Retention times, peak areas, chromatographic quality and ion ratios were all within acceptable limits. However, a quantitative interference was detected, which produced a % bias of more than ±20% for the 10 ng/mL suvorexant control. Extracts were evaluated using the MS^2 scan mode in order to identify which coeluting drug was responsible for the interference. These were attributed to sertraline (RT 1.4 min) and propoxyphene (RT 1.3 min). Although coelution of these drugs with the IS (1.4 min) did not impact the quality of the MRM transitions or chromatographic peak, they were responsible for a significant positive quantitative bias, likely due to the reduced ionization efficiency of the internal standard. This quantitative interference was explored further using positive drug controls (100 ng/mL suvorexant and IS) in the presence of ten-fold lower, equivalent, and ten-fold higher concentrations of propoxyphene and sertraline in triplicate.

The magnitude of the quantitative interference at a 1:10, 1:1 and 10:1 ratio of interferent to internal standard is shown in **Figure 3.7**. Unacceptable positive bias (24% and 22%) was observed for sertraline at equivalent and ten-fold higher concentrations, relative to the IS. Quantitative bias due to propoxyphene was even more pronounced, exceeding acceptable thresholds under all conditions tested (32 to 95%). Notably, the magnitude of the positive bias increased with increasing concentration of the interferent. This can be attributed to the reduced ionization efficiency of the IS due to the presence of the coeluting species, the competition for charge within the droplet, and the capacity-limited ionization within the ESI source itself.

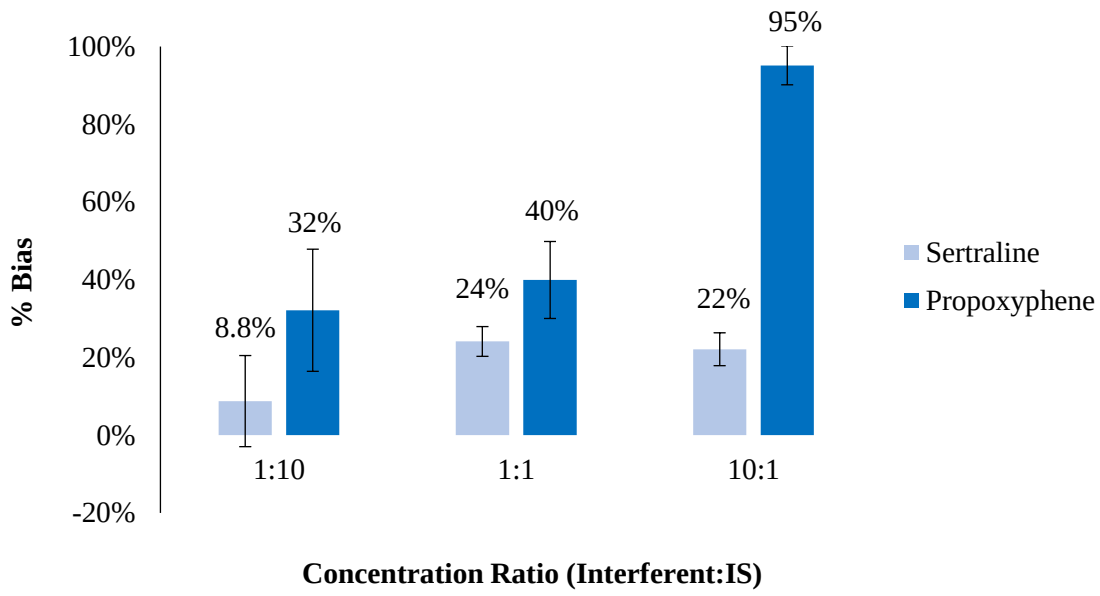


Figure 3.7. Systematic bias caused by coelution drugs. Bias was evaluated using interferent: internal standard (IS) concentration ratios of 1:10, 1:1 and 10:1 for sertraline and propoxyphene (n=3).

Overall Assay Performance

Validation parameters for the LC-Q/TOF-MS assay were previously described (19). Limits of detection and quantitation using the LC-MS/MS assay were 0.1 ng/mL and

0.5 ng/mL, respectively. Extracted ion chromatograms for suvorexant at the LOD are depicted in **Figure 3.8**. Precision and bias at the LOQ were 8.2% (n=18) and -7%. The calibration model was selected using various weighting options, residual plot analysis, and coefficients of determination. Although the LOQ was 0.5 ng/mL, a calibration range of 2 – 200 ng/mL was selected for routine analysis using a weighted (1/X) quadratic model. Coefficients of determination (R^2) ranged from 0.991-0.999 with an average R^2 value of 0.997 over five days. Intra- and inter-assay precision ranged from 4-7% (n=5) and 7-9% (n=15), respectively, using one-way ANOVA (**Table 3.4**). No carryover was observed.

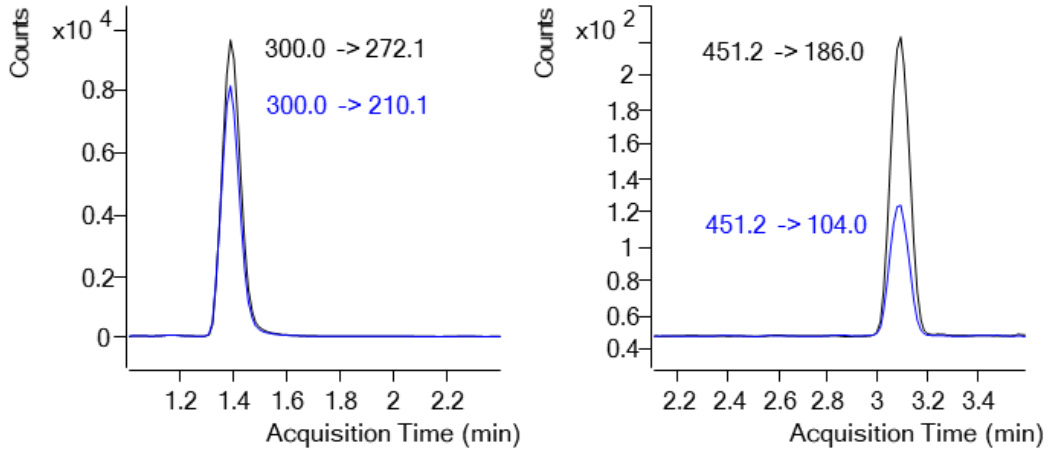


Figure 3.8. Representative extracted ion chromatograms of IS (100 ng/mL) and suvorexant in a blood extract at the limit of detection (0.1 ng/mL).

Table 3.4. Precision and bias at low (5 ng/mL), medium (50 ng/mL), and high (160 ng/mL) concentrations.

Concentration (ng/mL)	Intra-assay CV (%) n=5	Inter-assay CV (%) n=15	Bias (%) n=15
5	7%	8%	0%
50	5%	9%	2%
160	4%	7%	-2%

Differences in LC-MS/MS and LC-Q/TOF-MS assay performance are summarized in **Table 3.5**. Although the LC-MS/MS method had increased sensitivity with a LOD of 0.1 ng/mL, LOQs using both methods were 0.5 ng/mL in whole blood. No appreciable differences were observed in terms of precision and bias, and following adjustment of the source conditions, matrix effects using the LC-MS/MS were completely mitigated. The side-by-side comparison of assay performance using both instruments confirms the experience in our laboratory, that quantitative targeted assays using LC-Q/TOF-MS are indeed comparable to LC-MS/MS.

Table 3.5. Summary of assay performance for the LC-MS/MS method and the previously validated LC-Q/TOF-MS method (19).

Validation Parameter		LC-Q/TOF-MS	LC-MS/MS
LOD		0.5 ng/mL	0.1 ng/mL
LOQ		0.5 ng/mL	0.5 ng/mL
Calibration Model		2-200 ng/mL (1/X Quadratic)	2-200 ng/mL (1/X Quadratic)
Precision	Intra-assay (n=5)	4-10%	4-7%
	Inter-assay (n=15)	5-13%	7-9%
Bias		-5-2%	-2-2%
Matrix Effects	Low (20 ng/mL)	16%	-8%
	High (100 ng/mL)	15%	-9%
Carryover		None	None

Discussion

During the validation of a quantitative assay for suvorexant in blood, significant differences in matrix effects were observed between LC-MS/MS and LC-Q/TOF-MS assays. Notably, software optimized source conditions that maximize absolute signal intensity for precursor and product ions did not produce optimum overall assay performance. Matrix effects have been described as the “Achilles heel” of quantitative LC-MS based assays (23). Despite the fact that LC-MS/MS is often considered to be the

preferred technique for quantitative analyses in biological matrices, important considerations and limitations exist. LC-MS based techniques are often preferred for bioanalysis due to their sensitivity and selectivity. However, the impact of matrix effects on accuracy, precision and robustness of bioanalytical methods is an area of concern in quantitative analyses (23-25).

Matrix effects are observed when a coeluting species affects the ionization efficiency of the analyte. Kebarle and Tang were the first to describe matrix effects using electrospray ionization in 1993 (26). Although the exact mechanism is unknown, it is believed to originate from the competition between the analyte and the coeluting substance at the electrospray interface. Although these effects may not be evident from chromatographic responses, they can have a deleterious effect on both accuracy and sensitivity in quantitative analyses (23). Post-column infusion and post-extraction addition techniques can be used to visualize matrix effects and quantify their influence, respectively. Amelioration of these effects may involve changes to the sample extraction methodology, additional sample preparation steps, re-optimization of chromatographic separation, alteration of mobile phase additives, or use of a coeluting or stable isotope labeled internal standard. At the time of this study, a deuterated internal standard for suvorexant was not commercially available. Although standard addition techniques can also compensate for matrix effects, they are not widely used for forensic toxicology purposes due to multiple sampling and increased sample volumes.

At the electrospray interface a fine spray of highly charged droplets produce single or multiply charged species from an aqueous/organic liquid mixture. In the presence of heat and a strong electric field, gas phase ions are produced. Changes in ionization

efficiency related to matrix effects can occur in either the liquid phase or the gas phase: In the liquid phase, saturation of the ESI droplets with analyte at their surface can inhibit the ejection of ions trapped inside the droplet. Physico-chemical properties including surface activity and polarity influence the competition for limited charge or space within the droplet. Biological matrices with endogenous compounds with high basicity and surface activity can quickly saturate ionization, resulting in suppression. High concentrations of analyte can also increase viscosity and surface tension of the droplet, changing efficiency of their formation, evaporation, and the number of gas phase ions that reach the detector. Various gas phase mechanisms have also been proposed. Once in the gas phase, charge can be lost through neutralization reactions or charge transfer. In addition, ESI can be influenced by the type of instrumentation and ion suppression can vary significantly between different source geometries (i.e. Z-spray ion source, orthogonal spray ion source). Therefore, source design must be considered as variations in capillary diameter, distance from capillary tip to counter electrode, electrolyte formation, and the resulting droplet radius can greatly impact ion suppression between different instruments that employ ESI (27). APCI is less susceptible to ME than ESI techniques. During ESI, ionization takes place in the liquid phase and the ion is transferred to the gas phase in a charged state. During APCI the molecule is transferred to the gas phase in the neutral state and is subsequently ionized. As a result, APCI is not susceptible to any of the mechanisms that can influence liquid phase ionization suppression. Although enhancement of ionization is also possible, it is described with less frequency.

Decreases in ionization efficiency can be caused by endogenous or exogenous suppressors. Endogenous suppressors can include salts, surfactants, carbohydrates, lipids,

polar organic molecules, and other co-extractive compounds found in biological matrices (25). Exogenous suppressors are interfering substances that come from a source other than the biological matrix. They can include artifacts from sample preparation or chromatography, including plasticizers, phthalates, organic acids, buffers, other compounds or coeluting drugs. Residual matrix components and phospholipids in particular can be a significant source of bias and imprecision in quantitative analysis (24). This is highly relevant in forensic toxicology, particularly during multi-analyte or systematic toxicological analyses using complex biological matrices (28). Rapid LC-based methods where multiple drugs are simultaneously quantified without a coeluting or SIL-IS are most vulnerable, due to the capacity-limited nature of electrospray ionization.

At the time of development there was no commercially available deuterated analog for suvorexant which could have mitigated the ion suppression caused by matrix components and coeluting drugs. The study highlights the need to critically evaluate the potential for quantitative bias during drug interference studies, particularly if a SIL-IS is not available. The study also demonstrates that although additional sample preparation and chromatographic changes can be undertaken, these can be labor intensive and meet with limited success. In this study, unacceptable endogenous matrix effects were completely eliminated by modification of the source conditions. Source optimization is highly compound dependent, as well as instrument dependent. Differences in sheath gas temperature, flow, nebulizer pressure, and capillary and nozzle voltage can greatly influence ionization. This compound dependence can be exploited for the purpose of endogenous matrix interferences. Although this amounts to a detuning of the source, the assay may be more robust as a result (29,30). While this approach slightly decreased overall

intensity of the instrument response, the assay still demonstrated excellent limits of detection and quantitation (0.1 and 0.5 ng/mL, respectively).

References

1. Cox, C. D., Breslin, M. J., Whitman, D. B., Schreier, J. D., McGaughey, G. B., Bogusky, M. J., et al. (2010) Discovery of the dual orexin receptor antagonist [(7R)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl]methanone (MK-4305) for the treatment of insomnia. *Journal of Medicinal Chemistry*. **53**, 5320-5332.
2. National Institutes of Health (2005) NIH State-of-the-science conference statement on manifestations and management of chronic insomnia in adults. *NIH Consensus and State-of-the-Science Statements*. **22**, 1-30. Available from: https://consensus.nih.gov/2005/insomniastatement.pdf (accessed November 17, 2018).
3. U.S. Food and Drug Administration (2013) Suvorexant Tablets Insomnia Indication, Peripheral & Central Nervous System Drugs Advisory Committee Meeting, NDA 204569. Available from: https://wayback.archive-it.org/7993/20170405225048/https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAdvisoryCommittee/UCM352970.pdf (accessed November 17, 2018).
4. Bennett, T., Bray, D., and Neville, M. W. (2014) Suvorexant, a dual orexin receptor antagonist for the management of insomnia. *P & T: A Peer-Reviewed Journal for Formulary Management*. **39**, 264-266.

5. U.S. Drug Enforcement Administration (2014) Schedules of controlled substances: placement of suvorexant into Schedule IV. Final rule. *Federal Register*. **79**, 51243–51247.

6. Uemura, N., McCrea, J., Sun, H., Donikyan, M., Zammit, G., Liu, R., et al. (2015) Effects of the orexin receptor antagonist suvorexant on respiration during sleep in healthy subjects. *The Journal of Clinical Pharmacology*. **55**, 1093-1100.

7. Ciu, D., Cabalu, T., Lai Yee, K., Small, J., Li, X., Liu, B., et al. (2016) In vitro and in vivo characterisation of the metabolism and disposition of suvorexant in humans. *Xenobiotica*. **46**, 1-14.

8. Patel, K. V., Aspesi, A. V., and Evoy, K. E. (2015) Suvorexant: A dual orexin receptor antagonist for the treatment of sleep onset and sleep maintenance insomnia. *Annals of Pharmacotherapy*. **49**, 477-483.

9. Baselt, R.C. (2017) Suvorexant. In Disposition of toxic drugs and chemicals in man, 11th edition. Biomedical Publications, Seal Beach, CA, pp. 2005.

10. Waters, B., Hara, K., Ikematsu, N., Takayama, M., Matsusue, A., Kashiwagi, M., et al. (2018) Tissue distribution of suvorexant in three forensic autopsy cases. *Journal of Analytical Toxicology*. **42**, 276-283.

11. Carson, M., and Kerrigan, S. (2017) Quantification of suvorexant in urine using gas chromatography/mass spectrometry. *Journal of Chromatography B*. **1040**, 289-294.

12. Scientific Working Group for Forensic Toxicology (2013) Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method

Validation in Forensic Toxicology. *Journal of Analytical Toxicology*. **37**, 452-474.

13. Breidinger, S. A., Simpson, R. C., Mangin, E., and Woolf, E. J. (2015) Determination of suvorexant in human plasma using 96-well liquid–liquid extraction and HPLC with tandem mass spectrometric detection. *Journal of Chromatography B*. **1002**, 254-259.

14. ASB/ANSI 098 Standard for Mass Spectral Data Acceptance in Forensic Toxicology – Draft (2019). Available at https://www.nist.gov/sites/default/files/documents/2019/03/20/standard_for_mass_spec_spectral_data_acceptance_-_asb.pdf (accessed June 17, 2019).

15. Iqbal, M., Ezzeldin, E., Khalil, N. Y., Al-Rashood, S. T. A., and Al-Rashood, K. A. (2017) Simple and highly sensitive UPLC-ESI-MS/MS assay for rapid determination of suvorexant in plasma. *Journal of Analytical Toxicology*. **41**, 114-120.

16. Iqbal, M., Khalil, N. Y., Ezzeldin, E., and Al-Rashood, K. A. (2018) Simultaneous detection and quantification of three novel prescription drugs of abuse (suvorexant, lorcaserin and brivaracetam) in human plasma by UPLC–MS-MS. *Journal of Analytical Toxicology*. **43**, 203-211.

17. Iqbal, M., Ezzeldin, E., Khalil, N. Y., Alam, P., and Al-Rashood, K. A. (2019) UPLC-MS/MS determination of suvorexant in urine by a simplified dispersive liquid-liquid micro-extraction followed by ultrasound assisted back extraction from solidified floating organic droplets. *Journal of Pharmaceutical and Biomedical Analysis*. **164**, 1-8.

18. Sullinger, S., Bryand, K., and Kerrigan, S. (2017) Identification of suvorexant in urine using liquid chromatography-quadrupole/time-of-flight mass spectrometry (LC-Q/TOF-MS). *Journal of Analytical Toxicology*. **41**, 224-229.

19. Skillman, B., and Kerrigan, S. (2018) Quantification of suvorexant in blood using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry. *Journal of Chromatography B*. **1091**, 87-95.

20. Gosetti, F., Mazzucco, E., Zampieri, D., and Gennaro, M. C. (2010) Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*. **1217**, 3929-3937.

21. Furey, A., Moriarty, M., Bane, V., Kinsella, B., and Lehane, M. (2013) Ion suppression; A critical review on causes, evaluation, prevention and applications. *Talanta*. **115**, 104-122.

22. Berg, T., and Strand, D. H. (2011) 13C labelled internal standards—A solution to minimize ion suppression effects in liquid chromatography–tandem mass spectrometry analyses of drugs in biological samples? *Journal of Chromatography A*. **1218**, 9366-9374.

23. Taylor, P. J. (2005) Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry*. **38**, 328-334.

24. Chambers, E., Wagrowski-Diehl, D. M., Lu, Z., and Mazzeo, J. R. (2007) Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography B*. **852**, 22-34.

25. Trufelli, H., Palma, P., Famiglini, G., and Cappiello, A. (2011) An overview of matrix effects in liquid chromatography-mass spectrometry. *Mass Spectrometry Reviews*. **30**, 491-509.

26. Kebarle, P., and Tang, L. (1993) From ions in solution to ions in the gas phase - the mechanism of electrospray mass spectrometry. *Analytical Chemistry*. **65**, 972A-986A.

27. Ghosh, C., Shinde, C. P., and Chakraborty, B. S. (2012) Influence of ionization source design on matrix effects during LC–ESI-MS/MS analysis. *Journal of Chromatography B*. **893-894**, 193-200.

28. Peters, F. T., and Remane, D. (2012) Aspects of matrix effects in applications of liquid chromatography–mass spectrometry to forensic and clinical toxicology—a review. *Analytical and Bioanalytical Chemistry*. **403**, 2155-2172.

29. McGuffey, J. E., Wei, B., Bernert, J. T., Morrow, J. C., Xia, B., Wang, L., et al. (2014) Validation of a LC-MS/MS Method for Quantifying Urinary Nicotine, Six Nicotine Metabolites and the Minor Tobacco Alkaloids—Anatabine and Anabasine—in Smokers' Urine. *PloS One*. **9**, e101816.

30. Janusson, E., Hesketh, A. V., Bamford, K. L., Hatlelid, K., Higgins, R., and McIndoe, J. S. (2015) Spatial effects on electrospray ionization response. *International Journal of Mass Spectrometry*. **388**, 1-8.

CHAPTER IV

MOLECULAR LIPOPHILICITY OF SUVOREXANT USING EXPERIMENTAL AND THEORETICAL ESTIMATES: IMPLICATIONS FOR FORENSIC TOXICOLOGY

This dissertation follows the style and format of *The Journal of Analytical Toxicology*

Abstract

Suvorexant is a dual orexin receptor antagonist (DORA) that promotes sleep by reducing wakefulness and arousal. Relatively few published analytical methods describe the analysis of suvorexant in forensic casework. Information regarding its distribution or occurrence in specimens of forensic interest is lacking, and some of the physicochemical properties of the drug have not been fully investigated. The aim of this study was to investigate the molecular lipophilicity of suvorexant, identify the drug in a series of thirteen toxicological investigations, and discuss its potential for postmortem redistribution. Partition coefficients of suvorexant were determined using octanol/water and other aqueous systems using liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS). Experimentally determined Log P values were compared with those obtained using predictive computational software. Suvorexant was quantitatively determined in a series of thirteen forensic toxicology investigations. Toxicological specimens included antemortem and postmortem blood, plasma/serum and vitreous fluid. The experimentally determined Log P value for suvorexant was in close agreement with theoretical Log P values. Suvorexant was identified in antemortem and postmortem blood at concentrations of 3-42 ng/mL. Paired central (C) and peripheral (P) blood was obtained in two cases, yielding C/P ratios of 2.0 and 2.2, consistent with previously published reports (0.9-1.4). Furthermore, all concentrations were within the therapeutic or sub-therapeutic range. Despite its lipophilic nature, this neutral and heavily protein bound drug may not exhibit significant postmortem redistribution. Other drugs were present in all of the cases reported, most frequently with opioids (85%) and benzodiazepines (54%).

Keywords: Suvorexant, Lipophilicity, Log P, Blood, Postmortem toxicology, LC-Q/TOF-MS

MOLECULAR LIPOPHILICITY OF SUVOREXANT USING EXPERIMENTAL AND THEORETICAL ESTIMATES: IMPLICATIONS FOR FORENSIC TOXICOLOGY

Introduction

Suvorexant (Belsomra®) is among a novel class of dual orexin receptor antagonists that are prescribed to treat insomnia. The drug inhibits the neuropeptides orexin A and B that are produced by neurons in the hypothalamus. These neuropeptides (also known as hypocretin 1 and 2) control the wake-promoting centers of the brain. Suvorexant decreases arousal and wakefulness, therefore producing an indirect sleep-promoting effect. It's mechanism of action and pharmacodynamics are distinct from other classical hypnotic drugs. As the first drug in its class, it was approved by the Food and Drug Administration (FDA) in 2014 and became commercially available in 2015 (1). It is approved for use in Japan, the United States, Canada and Australia. In the US it is listed under Schedule IV of the Controlled Substances Act (2). As a sedative hypnotic, it is a drug of interest to the forensic toxicology community. However, there have been relatively few case reports involving the drug to date, perhaps due to the scope of testing that is performed during toxicological screening. Due to its relatively recent introduction, its role in forensic toxicology investigations is still unknown. Limited literature has been published regarding the distribution of suvorexant in toxicological specimens and there is a gap in the literature detailing the physicochemical properties of suvorexant that could help predict its behavior in biological specimens after death. As a sedative hypnotic, it is a drug of interest to the forensic toxicology community. However, there have been relatively few case reports involving the drug to date, perhaps due to the scope of testing that is performed during toxicological screening. Due to its relatively recent introduction, its role in forensic toxicology investigations is still unknown. Limited literature has been published regarding

the distribution of suvorexant in toxicological specimens and there is a gap in the literature detailing the physicochemical properties of suvorexant that could help predict its behavior in biological specimens after death.

The structure of suvorexant ([(7R)-4-(5-chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl]-[5-methyl-2-(triazol-2-yl)phenyl]methanone, MK-4305) is shown in **Figure 4.1**. Its molecular formula is $C_{23}H_{23}ClN_6O_2$ (450.9 g/mol) and it has a high boiling point (670°C). Chemically, it is an organochlorine compound that contains a 1,3-benzoxazole, diazepane, aromatic amide and triazole functionality.

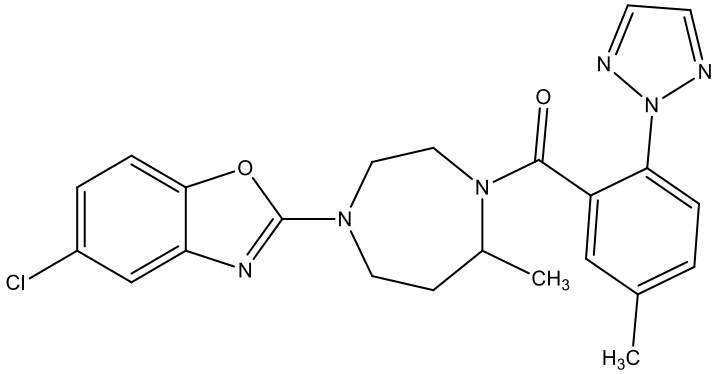


Figure 4.1. Chemical structure of suvorexant.

Peak plasma suvorexant concentrations are observed within two hours of oral administration (3). Following a 10-mg dose in healthy men (n=5), peak plasma concentrations (C_{max}) were 0.44 µM (198 ng/mL) (4). The FDA reports accumulations of 1- to 2-fold with once-daily dosing. Steady-state is achieved within 3 days and the mean half-life was approximately 12h (95% CI: 12 to 13) (1). The drug is extensively bound (>99%) to plasma proteins (serum albumin and α-1-acid glycoprotein) and oral bioavailability is reported to be ~82% (3).

The volume of distribution (V_d) for suvorexant has been reported to be 0.5-0.9 L/kg, and the FDA reported values between 49-105.9 L depending on the route of administration

(1, 5). The different units used to report V_d have caused inconsistencies among published reports for suvorexant (1, 6). The V_d is essentially a proportionality constant that relates the amount of drug in the body to the concentration of the drug in the plasma at equilibrium. Drugs that are highly bound to plasma proteins but not to tissue components tend to have low volumes of distribution. Conversely, those that accumulate in organs due to active transport or by specific binding to tissue molecules have high volumes of distribution, which can exceed the anatomical body volume. In clinical studies the V_d can be used to estimate the dose required to achieve a given plasma concentration. This is particularly important when peak plasma thresholds are necessary to achieve the therapeutic effect (as is often the case for hypnotic drugs). Drug dosage may be adapted accordingly and may need to consider changes in V_d due to individual height, weight, body mass and age. Distribution of drugs throughout the body is dependent on many factors including the lipophilicity of the drug, acid/base character, protein binding and transport mechanisms. In the postmortem period, distribution is further complicated by site and time-dependent variables.

While lipophilicity and the V_d can influence the tendency of a drug to exhibit PMR, many other factors are important. While antemortem samples can be among the most useful specimens for this purpose, they are not always available. Drug lipophilicity plays a key role in the interaction between compounds and receptors as well as other macromolecules, some of which may constitute biological membranes (7). Food and drugs are highly dependent on permeability and solubility to reach their target site in the body, both of which are influenced by the lipophilicity of a compound. In addition, metabolism or biotransformation of a compound can alter its properties such as size, mass, charge, and

lipophilicity (8). Measuring lipophilicity can provide information on a drug's affinity for lipid environments and can be measured in a number of ways (9).

Molecular lipophilicity is perhaps one of the most important physicochemical properties of a drug. It influences solubility, absorption, distribution, central nervous system (CNS) penetration, plasma protein binding, and partitioning into tissues and organs. The lipophilicity of a species may be determined experimentally by measuring the differential solubility of a compound between two immiscible layers. The resulting ratio of concentrations of the compound in each phase is referred to as the partition coefficient (P), most often expressed as Log P. The most commonly used solvent systems are *n*-octanol and water. For ionizable substances, the drug may exist in a variety of species (charged and uncharged) at any given pH. In those instances, the distribution coefficient (D) is the most appropriate measurement, since it represents the differential solubility of all species (charged and uncharged) in the system. For ionizable species, Log P can be estimated from the calculated Log D value, as long as the pKa for the drug is known. Although both Log P and Log D describe the lipophilicity of a compound, Log D is a useful descriptor for ionizable species. Nevertheless, the parameter is useful in forensic toxicology when comparing molecular lipophilicity between species. Due to the logarithmic scale, a Log P value of 1 indicates a 10-fold preference for the organic phase, opposed to the aqueous phase. Even within a particular drug class, Log P values vary considerably. Among the opioids, Log P values range from <1 (e.g., oxymorphone) to 5 (e.g., methadone). Experimental measurement of Log P values can be determined using shake-flask, electrochemical, pH-metric and chromatographic-based methods. Alternatively, Log P can be predicted computationally, using a variety of software approaches.

There are many ways to experimentally determine Log P values for a compound either directly, or indirectly. The shake-flask method, filter probe, chromatographic, pH-metric and electroanalytical (potentiometric titration, cyclic voltammetry) techniques are widely used (8-14). The shake-flask method is a direct measurement that is considered to be among the most accurate (11). Using this approach, an organic/aqueous mixture (e.g., octanol/water) is shaken with analyte until equilibrium is achieved. The phases are subsequently separated, and the concentration of the analyte is then measured in each phase. Disadvantages of using this method are the special consideration of solvent volumes to ensure accurate determination of analyte concentration in each phase, the need for high purity of solutes and solvents, solubility of the analyte itself, and the formation of micro-emulsions (8, 9). Other drawbacks include its labor intensiveness, inaccuracy due to potential impurities, adsorption to glass walls, and for highly lipophilic compounds, very low concentrations in the aqueous phase (in the parts per billion range). However, the shake-flask method is considered the most reliable technique to measure the lipophilicity of compounds with Log P values ranging from -3 to 4, and is a recommended procedure due to its simplicity. Additionally, in order to model biological partitioning, different aqueous systems can be used, with different ionic strengths and pH values.

Computational methods based upon atom, fragment, electrotopological, and knowledge-based systems are also available. This theoretical approach can be extremely useful during the drug discovery process, or when very little is known about a substance (i.e., new psychoactive substances). In some computational models, the Log P value is calculated by determining the summation of hydrophobic contributions from each constitutive fragment of a molecule to equal the hydrophobicity of that molecule. As the

length of constituent groups increase or as branching increases, the Log P may also be reduced from the expected value. These factors as well as the addition of polar groups (such as H- or S-) are considered to compensate for the effects of hydrogen bonding and hydrophobic shielding. These additional considerations are included in a "correction factor" that can be incorporated into Log P estimations. Molecule structure, perception of features (number of each atom, number of each bond type, number of hydrogens attached to non-hydrogens, connections characteristics, functional groups, polar fragments, and ring information), and correction factors may be considered (7). ALogPS 2.1, ACD Labs/LogP and KowWin 1.67 are common computational methods for Log P prediction and these are explored in more detail in this study.

ALogPS 2.1 predicts theoretical lipophilicity of a compound in addition to its aqueous solubility. Lipophilicity values are predicted by evaluating electronic and topological characteristics of the molecule. Electrotopological-state (E-state) indices are assigned to each atom type and its neighboring atoms, and with associative neural network modeling that was developed by Tetko et al, log P values can be estimated (13-16). The KowWin software estimates Log P values through the use of the atom/fragment contribution method. Each atom or fragment contribution value is summed and then multiplied by the frequency each of those occurs in the molecule. A reported advantage of the KowWin estimation is that it incorporates a correction factor that takes into account the linear equation constant and steric interactions (13, 15). ACD Labs/LogP octanol-water partition coefficient values are estimated through the use of experimentally or statistically determined fragment contribution summations (17). ACD Labs/LogP also uses three different algorithms (Classic, GALAS, or consensus model) to help predict LogP based on

molecular fragmentation and structural factors (17,18). ACD/LogP uses the "additive-constitutive" algorithm which sums contribution from individual atoms, as well as fragments and their intramolecular interactions. This algorithm also takes into special consideration carbon atoms not within functional groups based on their hybridization and hydrogen bonds, and checks for the possibility of tautomerization (19). Although each computational approach has proven useful for predicting Log P values, some studies have shown that programs that using topological data combined with E-state indices may provide more consistent Log P value estimations (15).

Relatively few studies have described the analysis of suvorexant in biological matrices. Analytical methods for the quantitation of suvorexant in a variety of fortified matrices have been published using multiple platforms. Quantitation of suvorexant in urine has been reported using gas chromatography-mass spectrometry (GC-MS), LC-Q/TOF-MS and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (20-22). Methods have also been described for plasma using LC-MS/MS with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) (23-25). Only three published methods describe the quantitation of suvorexant in whole blood (one of the most frequently encountered forensic matrices) using LC-Q/TOF-MS and LC-MS/MS (26-28). In a series of case reports from Japan, Waters described the analysis of suvorexant in postmortem specimens from three autopsy cases, including tissues (27). As the only published case report to date involving suvorexant, relatively little is known regarding the distribution of suvorexant postmortem, or its role in forensic toxicology investigations.

This study further explores the physicochemical properties of the drug and presents twelve additional medicolegal death investigations (MDIs) and one antemortem

investigation where suvorexant was identified. Molecular lipophilicity was studied using both experimental and computational methods. A previously published and validated method was used to detect suvorexant in antemortem and postmortem toxicology specimens using LC-Q/TOF-MS (26). Toxicological findings were compared to those described by Waters et al, and the potential for postmortem redistribution is discussed (27).

Materials and Methods

Chemicals and Reagents

Suvorexant was obtained as a powder from Cayman Chemical (Ann Arbor, MI) and estazolam-D5 (0.1 mg/mL) was obtained from Cerilliant (Round Rock, TX). *N*-butyl chloride (99+% pure, 1-chlorobutane, Acros Organics) and Optima LC/MS grade acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). Formic acid (>95%) was obtained from Sigma-Aldrich (St. Louis, MO). LCMS grade methanol, concentrated hydrochloric acid (36.5-38.0%), sodium borate, and dibasic sodium phosphate were purchased from J.T. Baker (Center Valley, MA). Monobasic sodium phosphate, acetic acid (glacial), boric acid (granular), sodium acetate, and sodium chloride were obtained from Mallinckrodt Chemicals (St. Louis, MO). Potassium chloride, monobasic potassium phosphate, and *n*-octanol (>99.5%, TCI America) were obtained from VWR (Radnor, PA). All deionized water was produced in-house using a Millipore Direct-Q® UV Water Purification System (Billerica, MA). Drug-free bovine blood treated with 0.2% potassium oxalate (w/v) and 1% (w/v) sodium fluoride was received from QuadFive (Ryegate, MT). Adjudicated case specimens including antemortem blood, postmortem heart blood, postmortem peripheral blood, serum/plasma, and vitreous humor were analyzed.

Instrumentation

An Agilent 1290 Infinity Binary LC coupled to an Agilent 6530 Accurate Mass Quadrupole Time-of-Flight LC/MS (Santa Clara, CA) was used for the identification of suvorexant in toxicology specimens and for the determination of suvorexant partition coefficients. The targeted LC-Q/TOF-MS assay utilized positive electrospray ionization (ESI) and targeted acquisition (26). The MS scan range was 100-1600 amu, at a MS scan rate of 5 spectra/second, acquisition time of 200 ms/spec and mass tolerance of ± 5 ppm. Two product ions transitions were used for suvorexant (m/z 451.1664 > 186.0664 and 451.1664 > 104.0493) and the internal standard (IS) estazolam-D5 (m/z 300.1059 > 272.0875 and m/z 300.1059 > 210.1076). Quantitation ions are underlined. Collision energies of 50 and 30 eV were used for suvorexant and the IS, respectively. Ionization conditions were as follows: 150 eV fragmentor voltage, 2000 V nozzle voltage, 3000 V capillary voltage, 45 psi nebulizer pressure, 300°C drying gas temperature, 13 L/min drying gas flow, 350°C sheath gas temperature, and 12 L/min sheath gas flow. The mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B). Separation was achieved with gradient elution using a Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm particle size) and Poroshell 120 EC-C18 guard column (2.1 x 5 mm, 2.7 µm particle size) at 35°C. Using a flow rate of 0.4 mL/min, gradient elution was as follows: 40-80% B (0-3 mins); hold 1 min; decrease to 40% (5 mins), followed by post-equilibration (2 minutes).

Determination of Partition Coefficients

Theoretical partition coefficients (Log P) were calculated for suvorexant using three predictive software packages: ALogPS version 2.1, ACD Labs/LogP, and KowWin

1.67. Experimental partition coefficients were determined using the classical shake-flask method with *n*-octanol and water, in addition to other aqueous buffer/octanol systems with varying ionic strengths and pHs (**Table 4.1**). The aqueous systems that were used for Log P determinations included 10 mM hydrochloric acid (pH 2), 10 mM acetate buffer (pH 5), 10 mM phosphate buffer (pH 7.4), 100 mM phosphate buffer (pH 7.4), 10 mM phosphate buffered saline (pH 7.4), 200 mM phosphate buffered saline (pH 7.4) and 10 mM borate buffer (pH 8 and 9). Aqueous suvorexant stock solutions were prepared at concentrations of 10 and 100 µg/mL. To measure partitioning of suvorexant between aqueous and organic phases, 900 µL of deionized water (or buffer), 100 µL suvorexant stock solution (100 µg/mL), and 1000 µL of *n*-octanol were added to 10-mL screw-top, round-bottom glass centrifuge tubes (n=3). The proportion of drug in the octanol layer was measured indirectly using aqueous controls (no octanol). Aqueous controls (n=3) were prepared by adding 900 µL of deionized water (or buffer) and 100 µL suvorexant stock solution (10 µg/mL) in glass centrifuge tubes. Samples were rotary mixed for 5 minutes at 45 rpm, followed by centrifugation for 5 minutes at 4000 rpm. The *n*-octanol layer was then discarded and 250 µL of the aqueous fraction was transferred to a new tube. The aqueous fraction was then diluted 1:1 with 250 µL mobile phase, and 250 µL of this dilution was subsequently transferred to vials for LC-Q/TOF-MS analysis. The aqueous controls were prepared similarly by performing 1:1 dilution of control (250 µL) with mobile phase before transferring 250 µL of this solution to LC vials. Log P was determined by comparing the abundance of suvorexant in the octanol and aqueous layers (accounting for the ten-fold dilution factor) using the following equation:

$$LogP = Log\left(\frac{[suvorexant]_{octanol} * 10}{[suvorexant]_{aqueous}}\right)$$

Statistical analyses using one-way analysis of variance (ANOVA) and t-tests were performed to identify any significant differences in partitioning using the various aqueous systems. Results were compared to determine the effect of pH and ionic strength on the distribution of suvorexant between aqueous and organic phases.

Identification of Suvorexant in Authentic Case Samples

Suvorexant working solutions were prepared at concentrations of 0.02, 0.2, and 2 µg/mL in methanol. Drug-free whole blood was fortified with suvorexant over a calibration range of 2-200 ng/mL. Estazolam-D5 was prepared at a concentration of 2 µg/mL in methanol. Specimens from adjudicated casework were extracted using a previously validated extraction method for suvorexant in blood (26). For blood and vitreous specimens, 0.5 ml of sample was fortified with IS to achieve a final concentration of 100 ng/mL. Sodium acetate buffer, 1 mL (0.4M, pH 3.6) and *N*-butyl chloride (2.5 mL) were added before rotary mixing for 5 min and centrifuging at 3000 rpm (5 min). The organic layer was removed and evaporated to dryness under nitrogen at 50°C. Samples were reconstituted in 30 µL mobile phase A/B (50:50), transferred to LC vials and 2 µL was injected onto the LC-Q/TOF-MS for analysis. Using a weighted (1/x) calibration model, the limit of detection (LOD) and limit of quantitation (LOQ) in whole blood was 0.5 ng/mL (26).

Results and Discussion

Partition Coefficient Determination

Experimental partition coefficients were determined using a variety of octanol/aqueous systems in triplicate. These are depicted in **Figure 4.2** and **Table 4.1**. The octanol/water partition coefficient (Log P) was 3.45 ± 0.28, demonstrating the strong

tendency for suvorexant to favor organic solvent. Log P values at pH 2-10 ranged from 3.23- 4.04. One-way ANOVA (α= 0.01) indicated that there were no significant differences between any of the experimentally determined Log P values ($F(8,18)=3.54$, $p=0.01$). Log P values were not influenced by pH (2-10 at 10mM) ($F(5,12)=3.47$, $p=0.04$) at α= 0.01)) or ionic strength. Two-tailed t-tests revealed no significant differences between 10 and 100 mM phosphate buffer at pH 7.4 (α= 0.01) and phosphate buffer containing 10 or 200 mM saline (α= 0.01). This is consistent with its behavior as a neutral drug, despite its nitrogenous nature (20, 21).

Partition coefficients were also estimated using predictive software. The Log P values for ALogPS, ACD Labs/LogP and KowWin were 3.86, 3.62 ± 0.86, and 4.65, respectively (**Table 4.2**). The KowWin software uses atom/fragment contribution methods to estimate a theoretical octanol-water partition coefficient (13, 15). ACD Labs/Log P software uses a combination of algorithms to predict partition coefficients. These algorithms combined are based on thousands of experimental Log P values, isolation of carbons, and adjustment for data for similar compounds, while a consensus algorithm will weigh the calculated value to the best suited structure model. ACD/LogP values are determined based on molecular fragmentation and structural factors (17, 18). ALogPS software will predict the octanol/water partition coefficient through a combination of neural networks which will work in parallel to calculate a theoretical value and was trained on a database of thousands of molecules. These estimations are based on electronic and topological characteristics of a molecule, which may provide more consistency in theoretical Log P calculations (13, 15). ACD/LogP and ALogPS provided Log P values that were closest to the experimentally determined value. Theoretically determined values

using the shake-flask method were within 0.2 and 0.4 of the experimentally determined value (**Table 4.2**). This suggests that the summation of atom/fragment contribution and correction factors utilized by KowWin were not as effective compared with ACD/LogP and ALogPS.

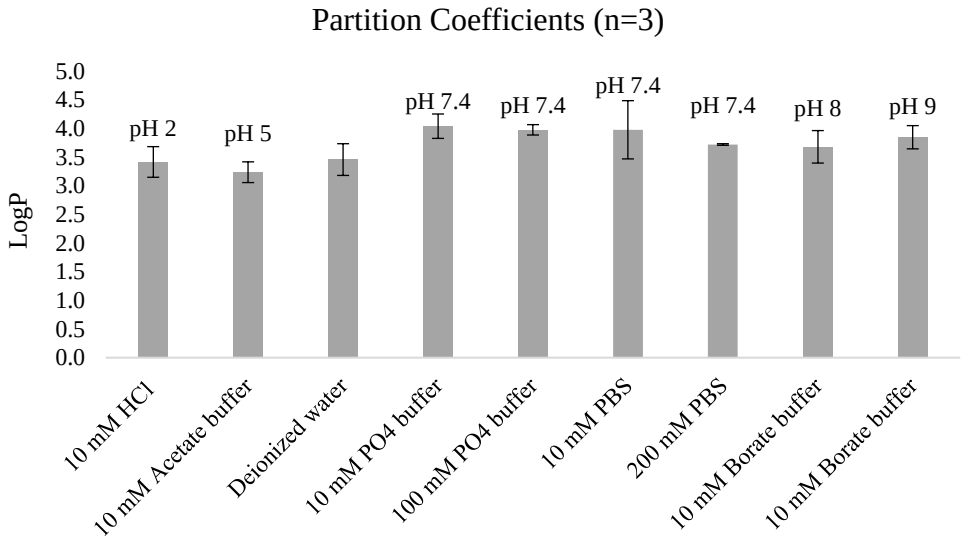


Figure 4.2. Partition coefficient (Log P) values for suvorexant in various aqueous systems (mean ± 1SD).

Table 4.1. Mean partition coefficients (n=3) for suvorexant using various aqueous systems.

Aqueous System	pH	Ionic Strength (mM)	Mean ± SD	%CV
Deionized water	**-**	**-**	**3.45 ± 0.28**	**8.0%**
10 mM HCl	2	10	3.41 ± 0.27	7.8%
10 mM Acetate buffer	4	10	3.23 ± 0.18	5.6%
10 mM Phosphate buffer	7.4	10	4.04 ± 0.21	5.3%
100 mM Phosphate buffer	7.4	100	3.97 ± 0.09	2.3%
10 mM PBS	7.4	200	3.97 ± 0.51	12.8%
200 mM PBS	7.4	3.72	3.72 ± 0.01	0.4%
10 mM Borate buffer	8	10	3.68 ± 0.28	7.7%
10 mM Borate buffer	9	10	3.84 ± 0.20	5.3%

Table 4.2. Comparison of experimental and theoretical Log P values for suvorexant.

Method	**Log P**	**ΔLog P (Thoeretical-Experimental)**	**Log P (Theoretical/Experimental)**
Experimental (Octanol/water)	3.45	NA	100%
ALogPS	3.86	0.41	112%
ACD Labs/LogP	3.62	0.17	105%
KowWin	4.65	0.79	135%

Suvorexant in Toxicology Investigations

Specimens from adjudicated casework were analyzed to determine the presence of suvorexant. These included twelve medicolegal investigations and one antemortem investigation involving a suspected impaired driver. Among the MDI cases, a total of four antemortem and thirteen postmortem specimens were evaluated, including both central and peripheral blood. **Table 4.3** summarizes the case information, including the source of the blood (when known). Concentrations in whole blood ranged from 3 to 42 ng/mL. Concentrations in postmortem specimens were well within the therapeutic range and the calibration range of the assay (2-200 ng/mL) (**Figure 4.3**). Peak plasma concentrations have been described for healthy men following 10, 50, and 100-mg oral doses of suvorexant by Sun et al, which resulted in C_{max} values of 198, 392, and 955 ng/mL, respectively (4). Suvorexant is currently available in doses of 5, 10, 15, and 20 mg, suggesting that peak plasma concentrations would typically be <200 ng/mL for patients prescribed the recommended 10-mg dosage (4). All postmortem samples tested demonstrated good chromatographic quality, acceptable ion ratios, and signal to noise ratios. Extracted ion chromatograms for a representative extract (Case# 6) are shown in **Figure 4.4**.

Table 4.3. Concentrations of suvorexant in forensic investigations.

Case #	Age	Investigation	Specimen	Tube	Concentration (ng/mL)	
1	25	Impaired Driving	AM Blood	Gray	3.2	Amphetamine < 50 ng/mL; Methamphetamine 145 ng/mL; Alprazolam 37 ng/mL; Morphine 11 ng/mL[1]; Trazodone present
2	45	MDI	Peripheral blood	Gray	3.5	Ethanol 0.018 g/dL; Morphine 427 ng/mL[1]; Diazepam 106 ng/mL; Nordiazepam 254 ng/mL; 7-Aminoclonazepam 30 ng/mL; Mirtazapine present; Sertraline present
			Heart blood	Gray	7	Ethanol 0.071 g/dL; Morphine 942 ng/mL[1]; 6-MAM present; Diazepam 113 ng/mL; Nordiazepam 194 ng/mL; 7-Aminoclonazepam 16 ng/mL; Mirtazapine present; Sertraline present
			Vitreous	Gray	ND	Ethanol not detected[2]; Morphine 48 ng/mL[1]; 6-MAM present
3	78	MDI	Peripheral Blood	Gray	16.8	Oxycodone 32 ng/mL[1]; Diphenhydramine 200 ng/mL; Fentanyl 5.4 ng/mL; Norfentanyl 0.68 ng/mL
			Heart Blood	Gray	36.5	-
4	51	MDI	AM Blood	Gray	21	Alprazolam 84 ng/mL; Hydrocodone 11 ng/mL; Bupropion 370 ng/mL; Hydroxybupropion 880 ng/mL; Ketamine 130 ng/mL; Norketamine 93 ng/mL; Metoprolol 1500 ng/mL; Ramelteon 15 ng/mL; Ramelteon M-II 21 ng/mL
			AM Plasma/Serum	Gold	3.6	-
5	50	MDI	Femoral Blood	Gray	36.8	Lamotrigine 10 mcg/mL; Levetiracetam 29 µ/mL; 10-Hydroxycarbazepine 92 µg/mL; Fluoxetine 100 ng/mL; Norfluoxetine 270 ng/mL; Mirtazapine 140 ng/mL; mCPP 150 ng/mL; Diphenhydramine 7700 ng/mL; Cyclobenzaprine 87 ng/mL
6	44	MDI	Cardiac Blood	Gray	13.2	Caffeine Positive; Cotinine Positive; Oxymorphone 400 ng/mL[1]; Paroxetine 240 ng/mL; Tramadol 43 ng/mL; O-Desmethyltramadol 22 ng/mL; Diphenhydramine 2500 ng/mL
7	62	MDI	Peripheral Blood	Gray	40.5	Caffeine Positive; Cotinine Positive; 7-Amino Clonazepam 38 ng/mL; Benzoylecgonine 330 ng/mL; Mirtazapine 6 ng/mL; Fentanyl 14 ng/mL; Norfentanyl 3.3 ng/mL

8	51	MDI	Femoral Blood	Gray	27.7	Caffeine Positive; Morphine 21 ng/mL[1]; Oxymorphone 5.1 ng/mL[1]; Levetiracetam 2.1 µg/mL; Venlafaxine 260 ng/mL; O-Desmethylvenlafaxine 950 ng/mL; Trazodone 0.30 µg/mL; Aripiprazole 68 ng/mL
9	36	MDI	Femoral Blood	Gray	15.6	Caffeine Positive; Naloxone Positive; Diazepam 420 ng/mL; Nordiazepam 460 ng/mL; Temazepam 29 ng/mL; Clonazepam 9.9 ng/mL; 7-Amino Clonazepam 110 ng/mL; Morphine 87 ng/mL[1]; Hydrocodone 34 ng/mL[1]; Oxycodone 430 ng/mL[1]; Oxymorphone 6.7 ng/mL[1]; Carisoprodol 5.7 µg/mL; Meprobamate 17 µg/mL; Sertraline 58 ng/mL; Desmethylsertraline 180 ng/mL; THCA 11 ng/mL; THC 2.1 ng/mL; Cyclobenzaprine 26 ng/mL; Fentanyl 1.6 ng/mL; Norfentanyl 0.45 ng/mL
10	51	MDI	Peripheral Blood	Gray	26.5	Caffeine Positive; Naloxone Positive; Amitriptyline 350 ng/mL; Nortriptyline 630 ng/mL; Hydroxyzine 200 ng/mL
11	33	MDI	AM Blood	Lavender	29.6	Caffeine Positive; Cotinine Positive; Duloxetine 93 ng/mL; Eszopiclone/Zopiclone 11 ng/mL
			AM Plasma/Serum	Green	12.9	
12	36	MDI	Peripheral Blood	Gray	17.2	Caffeine Positive; Naloxone Positive; Alprazolam 48 ng/mL; Benzoylecgonine 220 ng/mL; THCA 5.3 ng/mL; THC 0.79 ng/mL; Fentanyl 16 ng/mL; Norfentanyl 4.2 ng/mL
13	NK	MDI	Iliac Blood	Gray	41.5	Caffeine Positive; Lamotrigine 3.4 µg/mL; Quetiapine 17000 ng/mL; Yohimbine Positive

AM, antemortem; MDI, medicolegal death investigation; m-CPP, meta-chlorophenylpiperazine; THC, Δ-9-tetrahydrocannabinol; THCA, carboxy-THC; 6-MAM, 6-monoacetylmorphine; NK, not known; ND, not determined; [1]Free concentration; [2]Other drugs not detected or not tested (limited scope).

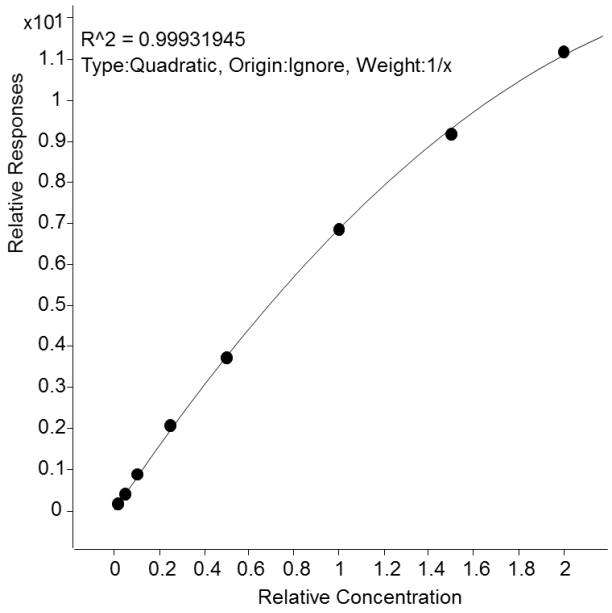


Figure 4.3. Representative calibration curve (2-200 ng/mL) in whole blood using the previously validated method (26).

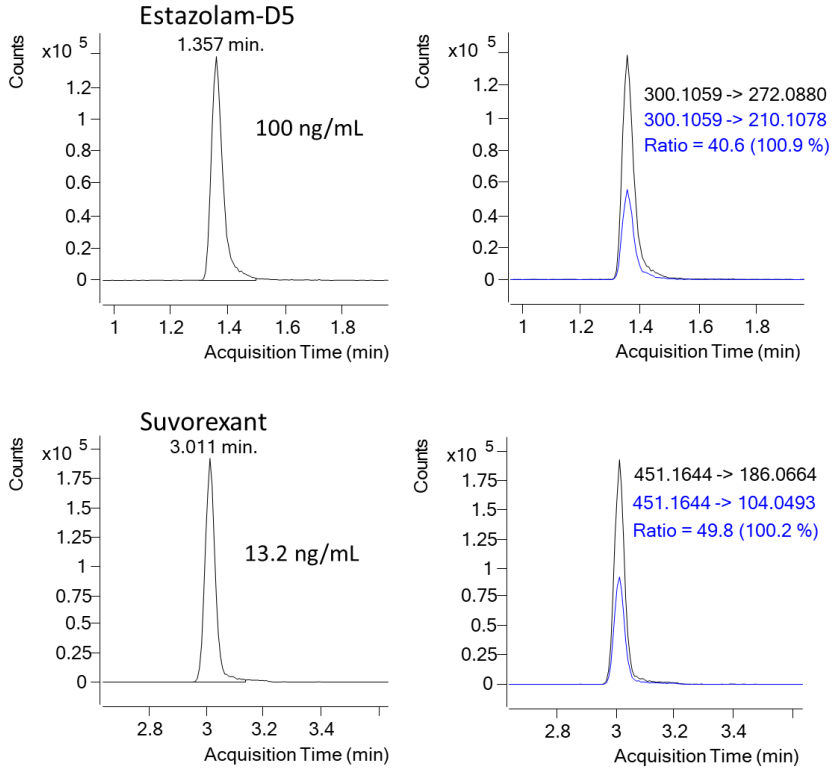


Figure 4.4. Representative extracted ion chromatogram (EIC) for suvorexant (13.2 ng/mL) and the IS (Case # 6).

Vitreous fluid was available in only one case. Although suvorexant was identified in both peripheral and heart blood, it was not identified in the vitreous, likely due to the very low drug concentration and poor partitioning due to its lipophilic character (**Table 4.2**). Suvorexant was identified in a total of seventeen specimens, including postmortem and antemortem blood, as well as serum/plasma. Blood/plasma ratios could not be determined due to hemolysis, and because the time of the antemortem blood and plasma collection was not known.

Suvorexant was identified in combination wide variety of other drugs, including amphetamines, cannabinoids, opioids, benzodiazepines and other therapeutics. None of the cases involved suvorexant alone. Opioids (11) and benzodiazepines (7) were the most commonly co-occurring substances, representing 85% and 54% of the suvorexant-positive cases. Other hypnotics or sedatives that are sometimes used as sleep aids (zopiclone, diphenhydramine, hydroxyzine) were identified in five cases. The mean age of the subjects was 46 (range 25-78). Suvorexant concentrations reported in **Table 4.3** represent the values obtained using the LC-Q/TOF-MS method described here. Suvorexant had been previously quantified in nine of the thirteen cases. The mean concentration upon reanalysis was in close agreement with original results (74%), despite refrigerated storage times of twenty months in some cases, and the absence of chemical preservative.

Paired central/peripheral blood samples were available in only two cases, yielding unremarkable C/P ratios of 2.0 and 2.2. This was consistent with the previous study by Waters et al, which reported C/P ratios of 0.9-1.4 (27). Waters described the distribution of drug in liver, kidney, spleen, pancreas, lung, muscle and fat. Also tested were right and left heart blood, left femoral blood, and urine. In all three cases, decedents had been

prescribed 20-mg tablets of suvorexant to be taken once nightly. The cause of death in the first case was determined to be drowning, but the decedent had levels of suvorexant in the blood samples ranging from 421-491 ng/mL. Drug concentrations in the fat were lower than blood (359 ng/g). The second case was determined to be a polydrug intoxication with blood levels ranging from 11-17 ng/mL, and highest tissue concentration of 40 ng/g was identified in the lung. The third case involved a suicide by hanging, with blood concentrations ranging from 138-155 ng/mL, and highest tissue concentration of 278 ng/g reported in fat (27). Notably, concentrations of suvorexant in urine were negligible or non-detectable. This might be attributed to the reported hydroxylation and extensive glucuronidation of the drug, which results in fecal elimination (66%) (3).

During clinical trials, gender and body mass index (BMI) were assessed in the pharmacokinetic models. In females, the area under the curve (AUC) and C_{max} were increased by 17% and 9% respectively following the 40 mg dose. Average concentrations of suvorexant approximately 9 hours after dosing were 5% higher among females. Although dose adjustments were not necessary based on gender alone, significant differences were observed in obese patients. Oral clearance is inversely related to BMI. In obese patients, the FDA reported AUC and C_{max} were increased by 31% and 17%, respectively. Mean concentrations 9 hours post-dose were 15% higher in patients with a BMI>30kg/m^2, compared to those within the normal BMI range (25 kg/m^2 or below) (1). As might be predicted, AUC and C_{max} in obese females were increased 46% and 25%, compared to non-obese females. Based upon the increased exposure among this group, the FDA recommends caution when considering increases in dose. No differences were observed between race and age (1).

Although suvorexant is a lipophilic drug, there is no evidence as yet to support significant postmortem redistribution. Many factors contribute to PMR including plasma protein binding, basicity, and lipophilicity. The study herein describes the analysis of suvorexant in 13 forensic toxicology investigations. The samples analyzed included antemortem and postmortem blood, serum/plasma and vitreous humor. The drug is reported to undergo metabolism to hydroxylated and glucuronidated species. However, there are no commercially available metabolites for suvorexant at this time, precluding their analysis in this study.

Only one case involved a living subject. The case involved a single-vehicle crash where the operator failed to maintain control of the vehicle and left their lane of travel. The driver's speech was slow, slurred, and at times incoherent. He was unsteady on his feet, appeared confused, had difficulty staying awake, and engaged in conversations with himself. His hand movements were shaky and eyelid tremors were present. The subject's 30 second time estimation was 50 seconds. Methamphetamine, alprazolam, morphine, trazodone and suvorexant were present in the blood (**Table 4.3**). Although the concentration of suvorexant was extremely low, its presence in a methamphetamine user is of note, possibly to offset the stimulant effects of the drug to prepare for sleep.

Conclusion

Suvorexant has not been widely reported in forensic investigations. Dose-related somnolence and CNS depression are the most common adverse effects associated with its use. It has also been shown to impair driving skills and may increase the risk of falling asleep while driving (6). Next-day impairments are found to be highest if suvorexant is taken with less than a full night of sleep remaining, with higher doses, or if co-administered

with other CNS depressants or CYP3A inhibitors. Although rare, parasomnias including sleep driving, preparing and eating food, making phone calls and other complex behaviors have been associated with hypnotics, including suvorexant (29). A dose-dependent increase in suicidal ideation has been observed, especially in patients with a previous diagnosis of depression (30). In this report, partition coefficients for suvorexant using experimental and computational methods were in good agreement. Of the thirteen cases presented, suvorexant concentrations were well within the therapeutic range. The vast majority of cases involved mixed drug intoxications, particularly involving opioids, benzodiazepines, or both. Despite its lipophilicity (Log P, 3.5), the V_d of suvorexant is low to moderate, and postmortem redistribution may not be a significant concern based on limited published reports to date.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures involving biological samples obtained from human decedents were in accordance with the ethical standards of the Sam Houston State University Institutional Review Board (Protection of Human Subjects Committee) in accordance with 45CFR46.101(b) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

1. U.S. Food and Drug Administration (2013) Suvorexant Tablets Insomnia Indication, Peripheral & Central Nervous System Drugs Advisory Committee Meeting, NDA 204569. Available from: https://wayback.archiveit.org/7993/20170405225048/https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAdvisoryCommittee/UCM352970.pdf (accessed November 17, 2018).
2. U.S. Drug Enforcement Administration (2014) Schedules of Controlled Substances: Placement of Suvorexant into Schedule IV. Final Rule. *Federal Register.* **79**, 51243-51247.
3. Ciu, D., Cabalu, T., Lai Yee, K., Small, J., Li, X., Liu, B., et al. (2016) In vitro and in vivo characterisation of the metabolism and disposition of suvorexant in humans. *Xenobiotica*. **46**. 1-14.
4. Sun, H., Kennedy, W.P., Wilbraham, D., Lewis, N., Calder, N., Li, X., et al. (2013) Effects of suvorexant, an orexin receptor antagonist, on sleep parameters as measured by polysomnography in healthy men. *Sleep*. **36**, 259-267.
5. Baselt, R.C. (2017) Suvorexant. In Disposition of toxic drugs and chemicals in man, 11th edition. Biomedical Publications, Seal Beach, CA, pp. 2005.
6. Merck & Co. (2014) Suvorexant prescribing information. Available from: http://www.merck.com/product/usa/pi_circulars/b/belsomra/belsomra_pi.pdf (accessed August 17, 2019).

7. Chou, J.T. and Jurs, P.C. (1979) Computer-assisted computation of partition coefficients from molecular structures using fragment constants. *Journal of Chemical Information and Computer Sciences*. **19**, 172-178.
8. Kempińska, D., Chmiel, T., Kot-Wasik, A., Mróz, A., Mazerska, Z. and Namieśnik, J. (2019) State of the art and prospects of methods for determination of lipophilicity of chemical compounds. *TrAC Trends in Analytical Chemistry*. **113,** 54-73.
9. Andrés, A., Rosés, M., Ràfols, C., Bosch, E., Espinosa, S., Segarra, V., et al. (2015) Setup and validation of shake-flask procedures for the determination of partition coefficients (logD) from low drug amounts. *European Journal of Pharmaceutical Sciences.* **76,** 181-191.
10. Pallicer, J.M., Calvet, C., Port, A., Rosés, M., Ràfols, C. and Bosch, E. (2012) Extension of the liquid chromatography/quantitative structure–property relationship method to assess the lipophilicity of neutral, acidic, basic and amphotheric drugs. *Journal of Chromatography A.* **1240**, 113-122.
11. Kah, M. and Brown, C.D. (2008) LogD: lipophilicity for ionisable compounds. *Chemosphere.* **72**, 1401-1408.
12. Donovan, S.F. and Pescatore, M.C. (2002) Method for measuring the logarithm of the octanol–water partition coefficient by using short octadecyl–poly(vinyl alcohol) high-performance liquid chromatography columns. *Journal of Chromatography A.* **952**, 47-61.
13. Medić-Šarić, M., Mornar, A., Badovinac-Črnjević, T., and Jasprica, I. (2004) Experimental and calculation procedures for molecular lipophilicity: A

comparative study for 3,3'-(2-Methoxybenzylidene)bis(4-hydroxycoumarin). *Croatica Chemica Acta.* **77**, 367-370.

14. Tetko, I.V. and Livingstone, D.J. (2007) 5.27 - Rule-Based Systems to Predict Lipophilicity. *Comprehensive Medicinal Chemistry II*. Taylor, J.B. and Triggle, D.J. Oxford, Elsevier**:** 649-668.
15. Medic-Saric, M., Mornar, A. and Jasprica, I. (2004) Lipophilicity study of salicylamide. *Acta Pharm.* **54**, 91-101.
16. Tetko, I.V. and Tanchuk, V.Y. (2002) Application of Associative Neural Networks for Prediction of Lipophilicity in ALOGPS 2.1 Program. *Journal of Chemical Information and Computer Sciences.* **42**, 1136-1145.
17. Petrauskas, A. and Kolovanov, E. (2000) ACD/Log P method description. *Perspectives in Drug Discovery and Design*. **19**, 99-116.
18. ACD/LogP. Available from: https://www.acdlabs.com/products/percepta/predictors/logP/ (accessed August 17, 2019).
19. Spessard, G.O. (1998) ACD Labs/LogP dB 3.5 and ChemSketch 3.5. *Journal of Chemical Information and Computer Sciences.* **38**, 1250-1253.
20. Carson, M. and Kerrigan, S. (2017) Quantification of suvorexant in urine using gas chromatography/mass spectrometry. *Journal of Chromatography B*. **1040**, 289-294.
21. Sullinger, S., Bryand, K. and Kerrigan, S. (2017) Identification of suvorexant in urine using liquid chromatography-quadrupole/time-of-flight mass spectrometry (LC-Q/TOF-MS). *Journal of Analytical Toxicology*. **41**, 224-229.

22. Iqbal, M., Ezzeldin, E., Khalil, N.Y., Alam, P. and Al-Rashood, K.A. (2019) UPLC-MS/MS determination of suvorexant in urine by a simplified dispersive liquid-liquid micro-extraction followed by ultrasound assisted back extraction from solidified floating organic droplets. *Journal of Pharmaceutical and Biomedical Analysis*. **164**, 1-8.
23. Breidinger, S.A., Simpson, R.C., Mangin, E. and Woolf, E.J. (2015) Determination of suvorexant in human plasma using 96-well liquid–liquid extraction and HPLC with tandem mass spectrometric detection. *Journal of Chromatography B.* **1002**, 254-259.
24. Iqbal, M., Ezzeldin, E., Khalil, N.Y., Al-Rashood, S.T.A. and Al-Rashood, K.A. (2017) Simple and highly sensitive UPLC-ESI-MS/MS assay for rapid determination of suvorexant in plasma. *Journal of Analytical Toxicology*. **41**, 114-120.
25. Iqbal, M., Khalil, N.Y., Ezzeldin, E. and Al-Rashood, K.A. (2018) Simultaneous detection and quantification of three novel prescription drugs of abuse (suvorexant, lorcaserin and brivaracetam) in human plasma by UPLC–MS-MS. *Journal of Analytical Toxicology*. **43**, 203-211.
26. Skillman, B., and Kerrigan, S. (2018) Quantification of suvorexant in blood using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry. *Journal of Chromatography B.* **1091**, 87-95.
27. Waters, B., Hara, K., Ikematsu, N., Takayama, M., Matsusue, A., Kashiwagi, M., et al. (2018) Tissue distribution of suvorexant in three forensic autopsy cases. *Journal of Analytical Toxicology*. **42**, 276-283

28. Skillman, B. and Kerrigan, S. (2019) Identification of suvorexant in blood using LC-MS/MS: Important considerations for matrix effects and quantitative interferences in targeted assays. *Journal of Analytical Toxicology*. (In press). DOI 10.1093/jat/bkz083.
29. Asai, Y., Sano, H., Miyazaki, M., Iwakura, M., Maeda, Y. and Hara, M. (2019) Suvorexant (Belsomra® Tablets 10, 15, and 20 mg): Japanese drug-use results survey. *Drugs in R&D*. **19**, 27-46.
30. Herring, W.J., Connor, K.M., Ivgy-May, N., Snyder, E., Liu, K., Snavely, D.B., et al. (2016) Suvorexant in patients with insomnia: Results from two 3-month randomized controlled clinical trials. *Biological Psychiatry*. **79**, 136-148.

CHAPTER V

DRUG-MEDIATED ION SUPPRESSION OF SUVOREXANT AND MITIGATION OF INTERFERENCES USING LIQUID CHROMATOGRAPHY-QUADRUPOLE/TIME OF FLIGHT MASS SPECTROMETRY (LC-Q/TOF-MS) AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1] Skillman, B., and Kerrigan, S. (2019). Submitted to *Journal of Chromatography B.*

Abstract

Liquid-chromatography mass spectrometry (LC-MS) is a powerful bioanalytical tool that is gaining widespread use in operational forensic toxicology laboratories. However, changes in ionization efficiency caused by endogenous or exogenous species must be carefully considered. While different modes of ionization can be used, electrospray ionization (ESI) can be especially prone to this phenomenon due to capacity-limited ionization. Decreased ionization efficiency of the target analyte or internal standard are possible in the presence of a competing coeluting compound during droplet desolvation in the source. This decreased ionization efficiency can influence the accuracy and sensitivity of analytical methods. While quantitative matrix effects are evaluated routinely during method development and validation, ion suppression arising from other drugs is not always assessed quantitatively, or in sufficient depth. In this study, the hypnotic drug suvorexant was used as a model compound for the investigation of such interferences. The potential for significant bias in quantitative analysis was demonstrated using this previously validated assay. Although stable isotope labeled internal standards can mitigate this issue, they are not always commercially available for new or emerging substances. In this study, quantitative biases due to ionization suppression are discussed, and techniques to overcome this challenge are presented.

Keywords: LC-Q/TOF-MS, LC-MS/MS, Ion suppression, Suvorexant, Interferences, Forensic toxicology

DRUG-MEDIATED ION SUPPRESSION OF SUVOREXANT AND MITIGATION OF INTERFERENCES USING LIQUID CHROMATOGRAPHY-QUADRUPOLE/TIME OF FLIGHT MASS SPECTROMETRY (LC-Q/TOF-MS) AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

Introduction

LC-MS techniques are increasingly used for a wide variety of applications, including forensic toxicology. LC-based methods facilitate the identification of thermally labile, polar, or nonvolatile analytes, without the need for derivatization (1, 2). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques have become the benchmark for bioanalytical assays due to their improved sensitivity and selectivity (3-5). High resolution mass spectrometry (HRMS), such as liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS), is also increasingly used. Despite their many advantages, performance of LC-MS based techniques can be negatively impacted by changes in ionization efficiency, including ion enhancement, or more frequently, ion suppression.

It was once commonly believed that LC-MS techniques would provide “unrivaled selectivity” using electrospray ionization (ESI) (1, 4). Electrospray is a technique that allows a wide variety of ion types to be transferred from the solution to the gas phase, enjoying widespread utility for biomolecules that sometimes exceed 100,000 Da (6). ESI is convenient in the sense that it uses conventional ionic solutions to transfer ions from the liquid to the gas phase, and the ionization of compounds can be altered by manipulating the solution chemistry (6). The ESI source contains a highly charged capillary which is

responsible for pneumatic manipulation of the solvent into a finely charged spray (7). The intensity of the spray is ultimately affected by source parameters such as the temperatures and gas flow rates used (7). Charged droplets are formed from electrolyte dissolved in a solvent which are subsequently evaporated, resulting in a smaller volume for the charges to disperse until fission occurs. The droplet fission process repeats until small, highly charged droplets are produced that are capable of transforming gas phase ions. Transfer to the gas phase occurs and ions are subsequently routed to the ion sampling region of the spectrometer (6).

Ion suppression is more likely to occur when multiple species are in the droplet in the absence of chromatographic resolution. The phenomenon becomes more problematic when minimal sample clean-up is performed, when the target analyte is only present in trace amounts, or when short run-times are used (i.e., fast LC) (1). Factors that increase the likelihood of ion suppression include high concentration, mass and basicity, as well as coelution with the analyte of interest (1). The total number of ions that can be formed during ESI is directly dependent upon the total surface area of all the droplets. Basicity and surface activity of coeluting compounds can determine their ionization efficiency. If the basicity or surface activity is higher for an interference than the analyte, and if it is present at sufficiently high concentration, the capacity to ionize all of the species in the droplet may be exceeded and the analyte signal is suppressed (1). Suppression is not only compound-dependent but can be caused by several mechanisms. One of the mechanisms by which ion suppression can occur is by competition between coeluting interfering ions and the target analyte for gas phase emission in the ESI source (1, 8, 9). In ESI, the analytes are introduced in the liquid phase. As the eluent is vaporized, the electrical density at the

surface of the droplet increases until it reaches its Rayleigh stability limit, which will then cause the droplet to divide into smaller droplets by electrostatic repulsion. This process will continue until the solvent has evaporated and the analyte ions enter the gas phase. However, when interferences are present in high concentrations this will increase the surface tension of the solvent droplet and result in reduced ability of the analytes to meet the droplet surface for ionization (1). Any analyte that is left within these droplets at the end of the fission process does not transfer to the gas phase and consequently does not make it through the detector. As such, the nature of ESI is capacity limited, and an excess of competing ions can result in ion suppression of the target analyte. As such, ion suppression can negatively influence the reliability of analytical results. In cases where internal standards are suppressed, an overestimation (positive bias) of the analyte concentration can result. Conversely, suppression of the analyte itself can produce a negative bias. Neither scenario is acceptable in forensic toxicology, where quantitative measurements may be relied upon. While it has been suggested that when an interference or matrix effect is detected, chromatography should be re-optimized to provide resolution between the interference and analyte, it is simply not always possible to achieve resolution from all known (and unknown) compounds or interferences. Such an approach may result in excessive run times, band broadening and decreased sensitivity. Therefore, a compromise is required.

Ion suppression is often highly dependent on the ionization source that is used. Atmospheric pressure chemical ionization (APCI) is less susceptible to some of the mechanistic ion suppressing events, as analytes are already in the gas phase prior to ionization (1, 10). However, APCI techniques are less commonly used than ESI, sometimes

due to their decreased analytical sensitivity. Source design and geometry can also affect ion suppression (1, 9, 11). Stable isotope labeled internal standards (SIL-ISs) are preferred for MS-based methods because they compensate for variations in extraction efficiency, chromatographic separation and detector sensitivity. More importantly, they are particularly useful for mitigating matrix effects and other interferences caused by capacity-limited ionization (12, 13). In theory, any coeluting internal standard should help compensate for ion suppression, this can be difficult to achieve without extensively shortening run times (13). With SIL-ISs, atoms in the analyte are replaced with stable isotopes such as ^{2}H and ^{13}C to give similar properties as the analyte and result in comparable retention times. Frequently, three or more atoms are replaced with its stable isotope, but unwanted resolution may occur with an increasing number of ^{2}H substitutes due to the mass difference between ^{1}H and ^{2}H being greater than the difference between ^{12}C and ^{13}C substitutions (14). Other factors to consider when choosing a labeled internal standard are the location of the substitutions on the analyte, the retention time, the molecular structure and weight, and the mobile phase that is used (14).

Matrix effects are the most commonly described cause of ion suppression or enhancement, but less frequently described matrix effects such as coeluting drugs can also be problematic when using ESI-based techniques. The mechanism for ion suppression in the presence of interfering compounds is much the same as with other endogenous matrix effects (lipids, extraction artifacts, additives), in that ions compete for transfer to the gas phase in the source, and such ionization is capacity limited. Possible sources of ion suppression (matrix or drug) must be thoroughly evaluated during method development, optimization and validation.

Interferences from coeluting species were previously explored for a suvorexant assay in whole blood using LC-Q/TOF and LC-MS/MS (15, 16). Suvorexant ([(7R)-4-(5-chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3,-triazol-2-yl)phenyl]methanone) (**Figure 5.1A**) is a sedative hypnotic drug that was recently introduced to the market in 2015, and resides under Schedule IV of the federal Controlled Substances Act (17, 18). Sensitive analytical methods are needed for its detection in forensic toxicology casework, particularly in impaired driving or drug-facilitated sexual assault (DFSA) investigations. We previously reported quantitative methods to identify suvorexant in whole blood using an acidic/neutral liquid-liquid extraction, followed by LC-Q/TOF and LC-MS/MS detection (15, 16). Both methods were validated in accordance with guidelines for forensic toxicology laboratories (19). The methods were both robust with LOQs of 0.5 ng/mL in whole blood, however quantitative interferences were observed in the presence of sertraline, a common drug that coeluted with the internal standard (estazolam-D5 (**Figure 5.1B**)). When assessing ion suppression or enhancement in LC-based quantitative methods, current guidelines state that potential interferences be analyzed in fortified samples, neat reference materials, or in previously analyzed case samples (19). While there are recommendations for assessing matrix effects (post-column infusion or post-extraction addition), there are no defined guidelines for assessing interferences from commonly encountered drugs or other exogenous compounds (3, 10). Drug interferences can be evaluated qualitatively or quantitatively, but there is no current requirement to assess the latter in forensic toxicology. Nevertheless, this is good laboratory practice to ensure the reliability of quantitative measurements. Although reports regarding signal suppression and enhancement have focused more on matrix effects than drug interferences,

the mechanism by which they influence the ionization is the same. These effects have been described as the “Achilles heel” of LC-MS based methods and although they can be minimized, there are no universal solutions at present (5, 9, 21).

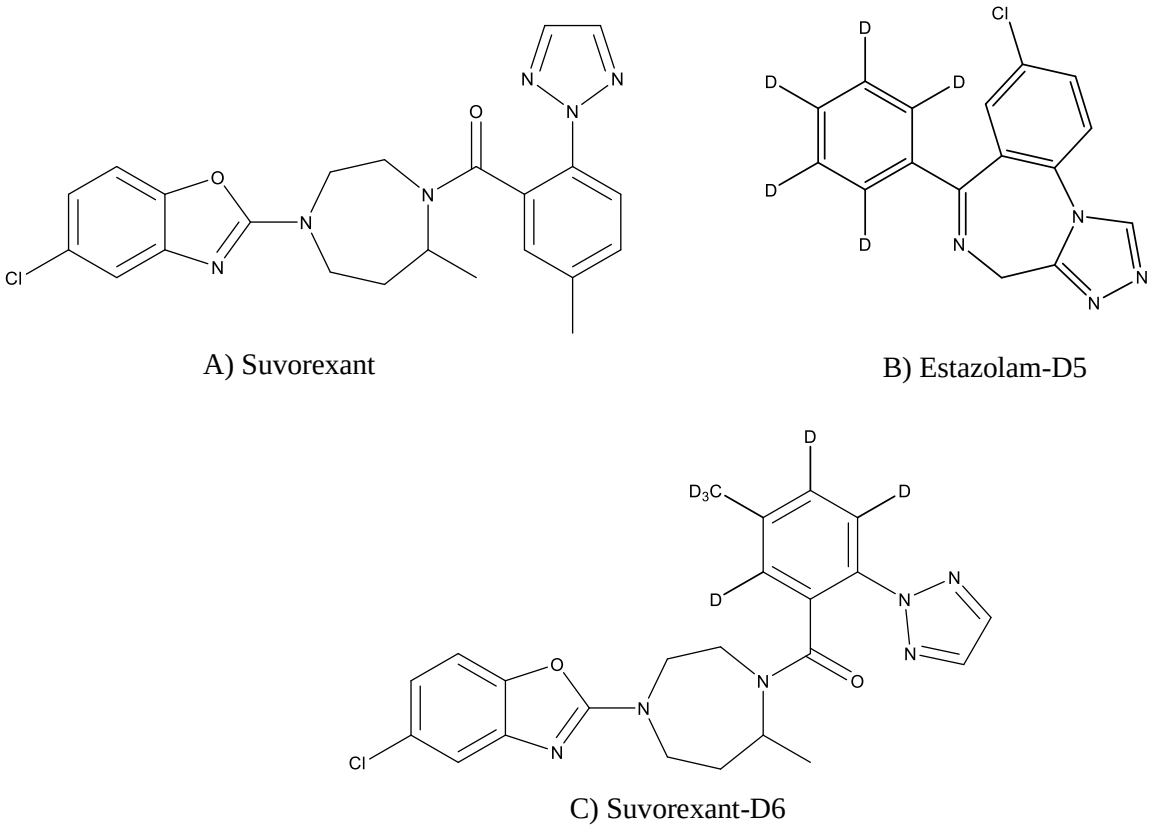


Figure 5.1. Structures of suvorexant (**A**), estazolam-D5 (IS) (**B**), and suvorexant-D6 (IS) (**C**).

The purpose of this study was to highlight these concerns and investigate possible ways to mitigate these issues. Interferences that arise as a product of capacity limited ionization in ESI methodologies can go undetected qualitatively (because retention time and ion ratios are unaffected), but still result in quantitative bias (4, 6, 20). This was the case for two previously reported suvorexant assays, for which there was no SIL-IS at the time of the development (15, 16). In this study, the mechanism of ion suppression was investigated using a statistical approach, and strategies for mitigating the effects of coeluting drug

interferences in the absence of isotope labeled internal standards are discussed. Subsequently, the use of deuterated suvorexant (suvorexant-D6 (**Figure 5.1C**)) was investigated, and comparisons are made with and without the use of a SIL-IS.

Materials and Methods

Chemical and Reagents

Suvorexant was purchased from Cayman Chemical (Ann Arbor, MI) as a powder. Estazolam-D5 and suvorexant-D6 internal standards were obtained from Cerilliant Corp. (Round Rock, TX) as methanolic standards (100 µg/mL). Fifty-two additional drugs (for the interference testing) were purchased from Cerilliant Corp. as listed in **Table 5.1**. Stock and working standards for all compounds were routinely prepared in methanol. The internal standard (IS) solution consisted of estazolam-D5 and suvorexant-D5 solutions (2 µg/mL) in methanol.

Table 5.1. Fifty-two common drugs used to evaluate qualitative and quantitative interferences in the detection of suvorexant from whole blood using LC-Q/TOF and LC-MS/MS.

Interference Study Drug Panel
Barbiturates Amobarbital, butalbital, pentobarbital, phenobarbital, secobarbital
Benzodiazepines 7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, clonazepam, diazepam, flurazepam, nordiazepam, oxazepam, temazepam
Z-Drugs Zaleplon, zolpidem, zopiclone
Common Drugs 11-nor-9-carboxy-delta-9-THC, acetaminophen, amitriptyline, amphetamine, bupropion, caffeine, carbamazepine, carisoprodol, cocaine, codeine, cyclobenzaprine, dextromethorphan, fluoxetine, gabapentin, hydrocodone, hydromorphone, ketamine, MDMA, meperidine, meprobamate, methadone, methaqualone, morphine, oxycodone, oxymorphone, phencyclidine, phenytoin, propoxyphene, pseudoephedrine, salicylic acid, sertraline, THC, tramadol, trazadone, valproic acid

MDMA, methylenedioxymethamphetamine; THC, tetrahydrocannabinol.

Sodium acetate (ACS grade) and glacial acetic acid (ACS grade) were obtained from Mallinckrodt Chemicals (St. Louis, MO). *N-butyl* chloride (Acros Organics, 99+% pure), Optima LC-MS grade formic acid, and Optima LC-MS grade acetonitrile were obtained from Fisher Scientific (Pittsburg, PA). LC-MS grade methanol was acquired from J.T. Baker (Center Valley, MA). All deionized water was purified in-house using a Millipore Direct-Q® UV Water Purification System (Billerica, MA). Whole drug-free bovine blood containing 1% sodium fluoride (w/v) and 0.2% potassium oxalate (w/v) was purchased from QuadFive (Ryegate, MT).

Instrumentation

Separation of analytes was achieved using two Agilent 1290 Infinity Binary LC systems (Santa Clara, CA) with identical LC conditions. For both platforms, matching Poroshell EC-C18 columns (2.1 x 100 mm, 2.7 µm particle size) and guard columns (2.1 x 5 mm, 2.7 µm particle size) were used. Mobile phases A and B were comprised of 0.1% formic acid in deionized water and 0.1% formic acid in acetonitrile, respectively. Gradient elution at 35°C was performed at a 0.4 mL/min flow rate as follows: 40% B (0 min), 40-80% B (0-3 min), hold 80% B (1 min), decrease 80-40% B (until 5 min), followed by re-equilibration (15, 16).

HRMS acquisition was performed on an Agilent 6530 Accurate Mass Quadrupole Time-of-Flight LC/MS (Santa Clara, CA) with positive mode electrospray ionization (ESI) using a previously published method (15). Detection of analytes was performed in parallel by LC-MS/MS on an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA) in positive ESI mode as previously described (16). ESI conditions for both ionization sources were identical: 300°C drying gas (13 L/min), 350°C sheath gas (12 L/min), 45 psi

nebulizer pressure, 3000 V capillary voltage, and 2000 V nozzle voltage. LC-Q/TOF analysis was performed using targeted MS/MS data acquisition (100-1600 amu) with narrow isolation widths (1.3 amu). A mass tolerance of ± 5 ppm was used, with a MS scan rate of 5 spectra/sec and a 200 ms/spec acquisition time (15). LC-MS/MS data was acquired using dynamic multiple reaction monitoring (dMRM) mode, with 200 ms dwell time for all ion transitions, as previously described (16). Compound-specific parameters such as fragmentor voltage, ion transitions, and collision energies for collision induced dissociation (CID) for suvorexant and IS can be found in **Table 5.2**.

Table 5.2. LC-Q/TOF-MS and LC-MS/MS instrument-specific parameters for data acquisition in the quantitation of suvorexant and internal standard.

Instrument	*Analyte*	*Fragmentor (V)*	*Precursor Ion (m/z)*	*Product Ions (m/z)*	*CID (eV)*
LC-Q/TOF-MS	Suvorexant	150	451.1644	**186.0664**	50
				104.0493	50
	Estazolam-D5	150	300.1059	**272.0875**	30
				210.1076	30
	Suvorexant-D6	150	457.2020	**192.1032**	50
				110.0865	50
LC-MS/MS	Suvorexant	127	451.2	**186.0**	21
				104.0	73
	Estazolam-D5	140	300.0	**272.1**	24
				210.1	48

Identification of exogenous drug-mediated interferences was performed using the LC and source conditions described. The LC-Q/TOF method was adapted for auto MS/MS data acquisition with the following stipulations: 40-1000 amu MS scan range (absolute thresholds of 200 counts for MS and 5 counts for MS/MS), 10 spec/sec MS scan rate, 5 spec/sec MS/MS scan rate, MS/MS medium isolation width (4 amu), and fixed collision energies of 30, 40, and 50 eV. An absolute precursor threshold of 6000 counts was used (0.01% relative) with 2 max precursors per cycle. LC-MS/MS data acquisition was

performed using MS2 scan mode at 135V fragmentor voltage, 100-500 amu scan range, and 500 ms scan time. The step size was 0.1 amu and a time filter width of 0.07 min was used.

Extraction

Suvorexant and IS were isolated from whole blood using and acidic/neutral liquid-liquid extraction. Drug-free blood was fortified with internal standard (100 ng/mL) and suvorexant over the calibration range (2-200 ng/mL). Sodium acetate buffer (0.4 M, pH 3.6) (1mL) and N¬-butyl chloride (2.5 mL) were added, followed by rotation (5 min) and centrifugation (3000 rpm, 5 min). The supernatant was removed to conical tubes and evaporated to dryness under nitrogen stream (50°C). Reconstitution of sample was performed with 1:1 mobile phase A/B (30 µL). The injection volumes for LC-Q/TOF-MS and LC-MS/MS analysis were 2 µL.

Identification of Interferences

Interferences were evaluated both qualitatively and quantitatively during validation of the LC-Q/TOF-MS and LC-MS/MS methods (15, 16). In the absence of a stable isotope internal standard for suvorexant at the time of validation, estazolam-D5 was used as the internal standard during initial interference studies. Interferences were evaluated by fortifying negative (0 ng/mL), low (10 ng/mL), and high (100 ng/mL) suvorexant-positive samples with 52 common drugs (1000 ng/mL). Qualitative interferences were evaluated using peak shape, retention time and ion ratios for suvorexant and internal standard in the presence (and absence) of other drugs. Retention time acceptance was ±2% and ion ratios acceptance was ±20% of the verified standard. Quantitative interferences were evaluated by comparing calculated concentrations of suvorexant to the true value and assessing bias.

If a quantitative bias exceeding ±20% was observed, the source of the drug interference was identified using auto MS/MS acquisition (LC-Q/TOF) or MS2 scan mode (LC-MS/MS). Once identified, suspected sources of drug interference with retention times close to suvorexant or estazolam-D5 were further investigated. Isolated interferences were then analyzed at different concentration ratios (1:10, 1:1, and 10:1 interferent to analyte) to determine the magnitude of the quantitative bias.

Mitigation of Interferences

Following isolation of drugs responsible for quantitative interferences (sertraline and propoxyphene), different strategies for mitigating interferences were assessed. The sample blood volume was assessed at 0.25 mL, 0.5 mL, and 1 mL in the presence of sertraline (1:10, 1:1, and 10:1 to that of the IS) to determine the effect sample volume can have on quantitative bias. Additionally, LC injection volumes (at the same concentration ratios) were assessed at 0.5, 1, and 2 µL in an attempt to mitigate drug-mediated interferences. Neat and extracted samples containing suvorexant, IS, and sertraline were compared to determine differences in response in the absence of matrix and to further demonstrate capacity limitations in ESI. The internal standard response for extracted samples containing sertraline was also compared to IS response in calibration samples to determine significant differences in the presence of sertraline. The newly available suvorexant-D6 standard was added to the LC-Q/TOF-MS acquisition method and qualitative and quantitative drug interferences were evaluated again to determine interference mitigation capabilities of compound-matched stable isotope internal standards.

Dilution Integrity

Using LC-Q/TOF targeted MS/MS, dilution integrity was investigated for various dilutions without and without the use of suvorexant-D6. Suvorexant-positive blood stock was prepared at 160 ng/mL. Positive QCs were prepared using 0.5 mL whole positive blood and were fortified with either estazolam-D5 (n=4) or suvorexant-D6 (n=4) internal standard (100 ng/mL). Dilution integrity was investigated using 2-fold and ten-fold dilutions of blood. The appropriate volume of blood (0.25 mL or 0.05 mL) was diluted in either sodium acetate buffer (pH 3.6, 0.4M), or drug-free blood (0.25 mL or 0.45mL) to achieve a total diluted specimen volume of 0.5 mL. Dilutions were performed in two identical sets, and each set was fortified with respective internal standard (n=4). Results were compared with quantitative values in undiluted blood (n=4). Following fortification of internal standard, samples were subjected to LLE as described and quantitated using a weighted 1/x quadratic calibration model (2-200 ng/mL).

Results and Discussion

Identification of Interferences

During the initial method validation, both LC-Q/TOF-MS and LC-MS/MS methods were evaluated for qualitative interferences from 52 common drugs. No qualitative interferences were identified in either assay using negative and positive controls: peak shape, retention time and ion ratios for estazolam-D5 and suvorexant were all within acceptable criteria (**Figure 5.2**). However, other drugs were present at 10-fold and 100-fold higher concentration (relative to the analyte or IS) quantitative biases were observed using low (10 ng/mL) and high (100 ng/mL) suvorexant positive controls. Sources of drug interference were identified from precursor ions using full scan acquisition on the LC-

Q/TOF-MS. Extracted ion chromatograms for two drugs that coeluted with estazolam-D5 are shown in **Figure 5.3**. Although no interferences were identified for suvorexant, propoxyphene and sertraline elute very close to the internal standard. These were further investigated using whole blood fortified with an excess of drug relative to the IS. Sertraline, which coeluted with the internal standard, was identified as the quantitative interference using LC-Q/TOF-MS and was further investigated at 1:10, 1:1, and 10:1 sertraline:estazolam-D5 concentrations (n=3). It was determined that as the drug concentration increased, the magnitude of the positive bias also increased (83% at 10:1), exceeding acceptability limits in forensic toxicology (Figure 4) (15, 19). This positive bias in the quantitative result was attributed to the decrease in ionization efficiency of the IS. The same experiment was repeated during the cross-validation of the LC-MS/MS method. MS2 scan mode acquisition was used to identify the potential interferences. Again, no qualitative interferences were identified, however a quantitative interference was attributed to sertraline and propoxyphene, with the added interferent resulting from very slight retention time differences between the two EC-C18 columns used on each platform. The bias associated for sertraline was 24% at equivalent concentrations and 22% at 10-fold higher concentrations, whereas propoxyphene bias ranged from 32-95% for all concentrations used (**Figure 5.4**) (16). This further demonstrates the difficulty eliminating all interferences simply by altering chromatographic conditions, as one interference can easily be replaced by another due to small shifts in retention time. Although every effort is made to establish chromatographic methods that a fit for purpose, resolution of analyte or IS form every possible other drug or metabolite is not an achievable goal. Although this issue may be rare, it may be under-recognized if laboratories do not assess drug

interferences quantitatively. Moreover, the problem will be exacerbated if rapid-LC methods are utilized for quantitative determination.

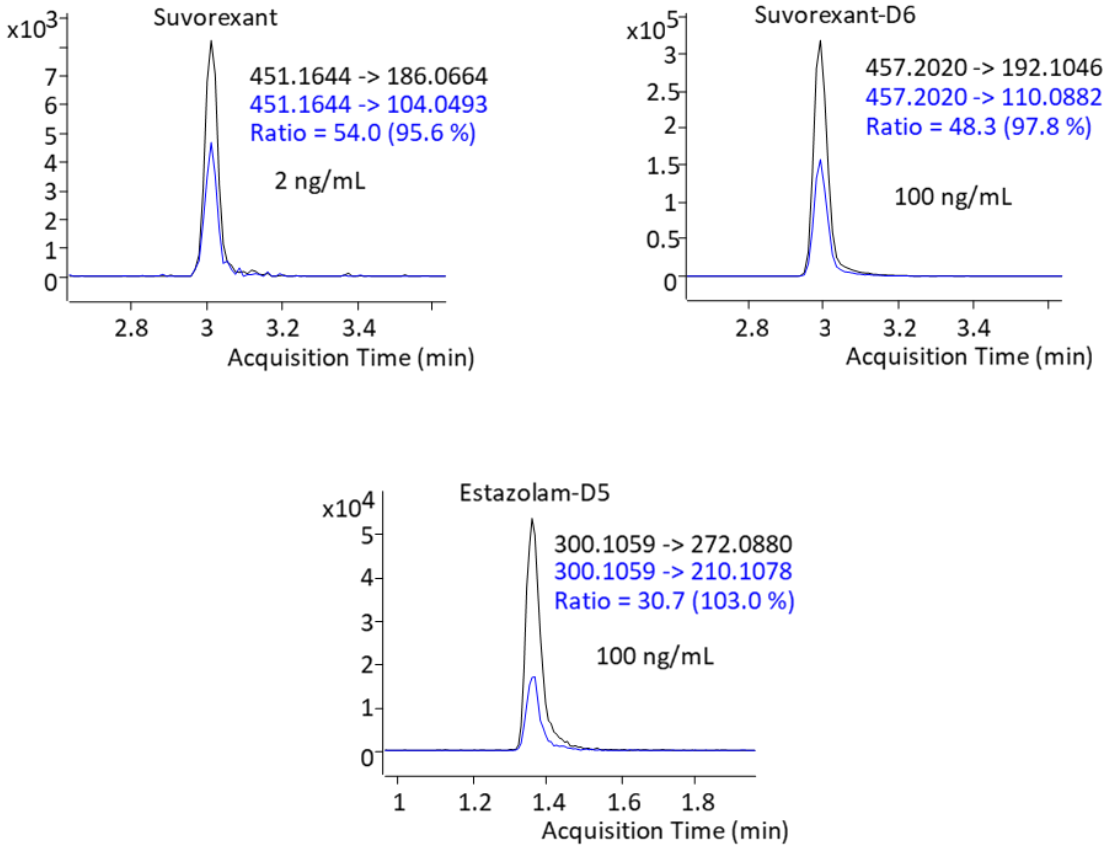


Figure 5.2. Extracted ion chromatograms (EICs) for suvorexant (100 ng/mL), estazolam-D5, and suvorexant-D6 in the presence of 52 common drug interferences.

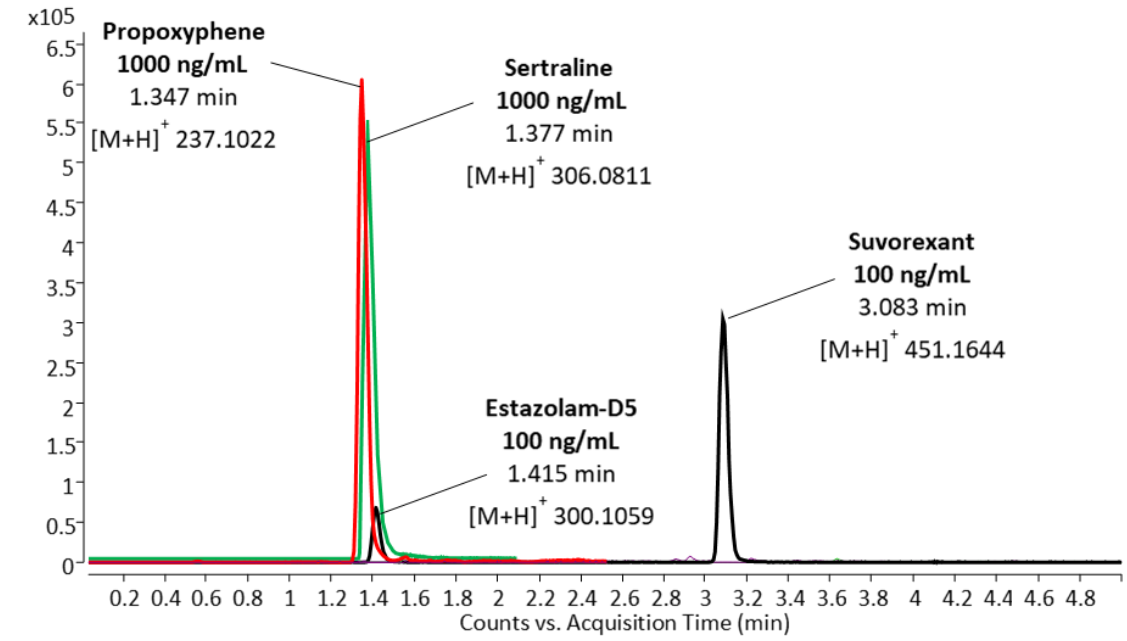


Figure 5.3. Extracted ion chromatogram (EIC) for interferences (1000 ng/mL) identified near the retention time of the IS (100 ng/mL).

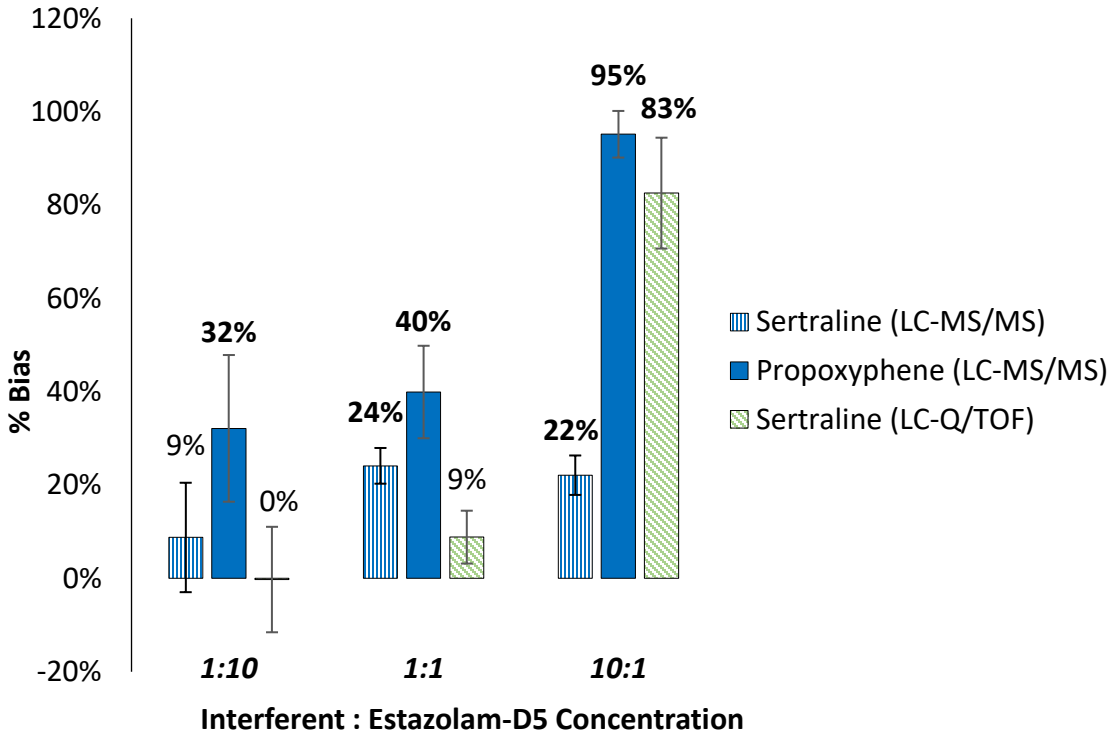


Figure 5.4. Systematic bias using ESI due to coelution of IS and interfering drugs at concentration ratios of 1:10, 1:1, and 10:1 interferent:estazolam-D5 (n=3, mean ± 1SD) (15, 16).

Using the LC-Q/TOF-MS assay, the absolute response of IS in the presence of sertraline at differing ratios (1:10, 1:1, and 10:1 sertraline:estazolam-D5) was statistically evaluated using one-way analysis of variation (ANOVA). Results were found to be significantly different ($F(3,15)= 22.9$, $p<0.001$, $\alpha=0.05$). Two-tailed t-tests showed no differences in response at 1:10 or 1:1 sertraline:estazolam-D5. However, a significant difference was observed between calibrator response and controls containing 10:1 sertraline:estazolam-D5 ($\alpha= 0.05$). The same was true for LC-MS/MS when responses were compared using ANOVA ($F(3,15)= 119.2$, $p<0.0001$, $\alpha= 0.05$). Two-tailed t-tests showed significant differences between calibrators and sertraline controls at 10:1 ($p<0.0001$, $\alpha=0.05$). While the overall decreased ionization efficiency of the internal standard in the source was clearly evident from the decreased response for estazolam-D5 in the presence of an excess of coeluting drug, this type of interference could go undetected for an analyte in a case sample where the expected response is unknown.

The effects of capacity limited ionization are only exaggerated in the presence of matrix as more species compete for access to the droplet surface during the ionization and gas-phase emission process in ESI. These endogenous species can increase viscosity of the solvent droplets and can also reduce efficiency with the presence of unionized precipitating species which can decrease desolvation. This phenomenon was shown when the absolute response of estazolam-D5 in the presence of sertraline was also compared between extracted samples and neat preparations (100 ng/mL IS and 1:10, 1:1, and 10:1 sertraline). Using two-tailed student t-tests, at 1:10 sertraline: estazolam-D5 there were no significant differences in LC-Q/TOF-MS response, however the internal standard response was significantly different between extracts and non-extracted samples at 1:1 ($p= 0.01$) and

10:1 ($p= 8.0 \times 10^{-3}$) at $\alpha= 0.01$. LC-MS/MS response was significantly different between extracted and neat samples at 10:1 ($p= 2.2 \times 10^{-3}$, $\alpha= 0.05$). While equivalent concentrations of sertraline and estzolam-D5 resulted in no analytical response differences between extracted controls and calibrators, as described, the estazolam-D5 response is significantly affected in the presence of matrix at this concentration when compared to a neat preparation. The matrix effect contributions to decreased ionization efficiency must also be considered when determining strategies for eliminating interferences.

Mitigation of Interferences

Once sertraline and propoxyphene were identified as the interferences responsible for unacceptable quantitative bias, several strategies for mitigation were explored. Sertraline was chosen as the model interferent due to its presence in both analytical methods (LC-Q/TOF-MS and LC-MS/MS) and subsequent experiments were performed in parallel on each instrument. The effect of sertraline on the quantitation of suvorexant was studied using varying sample volumes (0.25, 0.5, or 1 mL of whole blood) and LC injection volumes (0.5, 1, and 2 µL) in triplicate.

Using LC-Q/TOF-MS analysis, quantitative bias was within ±20% for samples that contained 10-fold lower (10 ng/mL) or equivalent (100 ng/mL) concentrations of sertraline to that of estzolam-D5 (100 ng/mL) using all sample volumes and injection volumes. However, as the concentration of sertraline was increased to 10-fold that of the IS, quantitative bias exceeding acceptability was observed at all sample volumes using the 2 µL injection volume (in the validated method). Using 0.5 mL whole blood (as required by the original extraction protocol), bias was mitigated by decreasing the LC injection volume to 0.5 µL. Bias could also be mitigated by using half the sample volume (0.25 mL blood)

in combination with injection volumes of 0.5 and 1 µL, but still exceeded ±20% at a 2 µL injection (**Figure 5.5A**). The same pattern was evident for the quantitative bias observed using LC-MS/MS at varying sample and injection volumes. Due to the overall increased sensitivity of the LC-MS/MS, bias could not be reduced to <20%, but consistent increases in bias were observed as the sample and injection volumes were increased. The magnitude of quantitative bias using 1 mL of sample (across all injection volumes) was large enough that it produced a relative suvorexant response that exceeded the upper limit of quantitation (ULOQ) (200 ng/mL) (i.e., >100% bias (**Figure 5.5B**). The same was true for the validated method protocol (0.5 mL blood, 2 µL injection) where bias exceeded 100% and the relative response for suvorexant was beyond that of the ULOQ (**Figure 5.5B**). These observations support the phenomenon of capacity limited ionization and its downstream effects on ionization efficiency resulting in ion suppression (5, 9, 21).

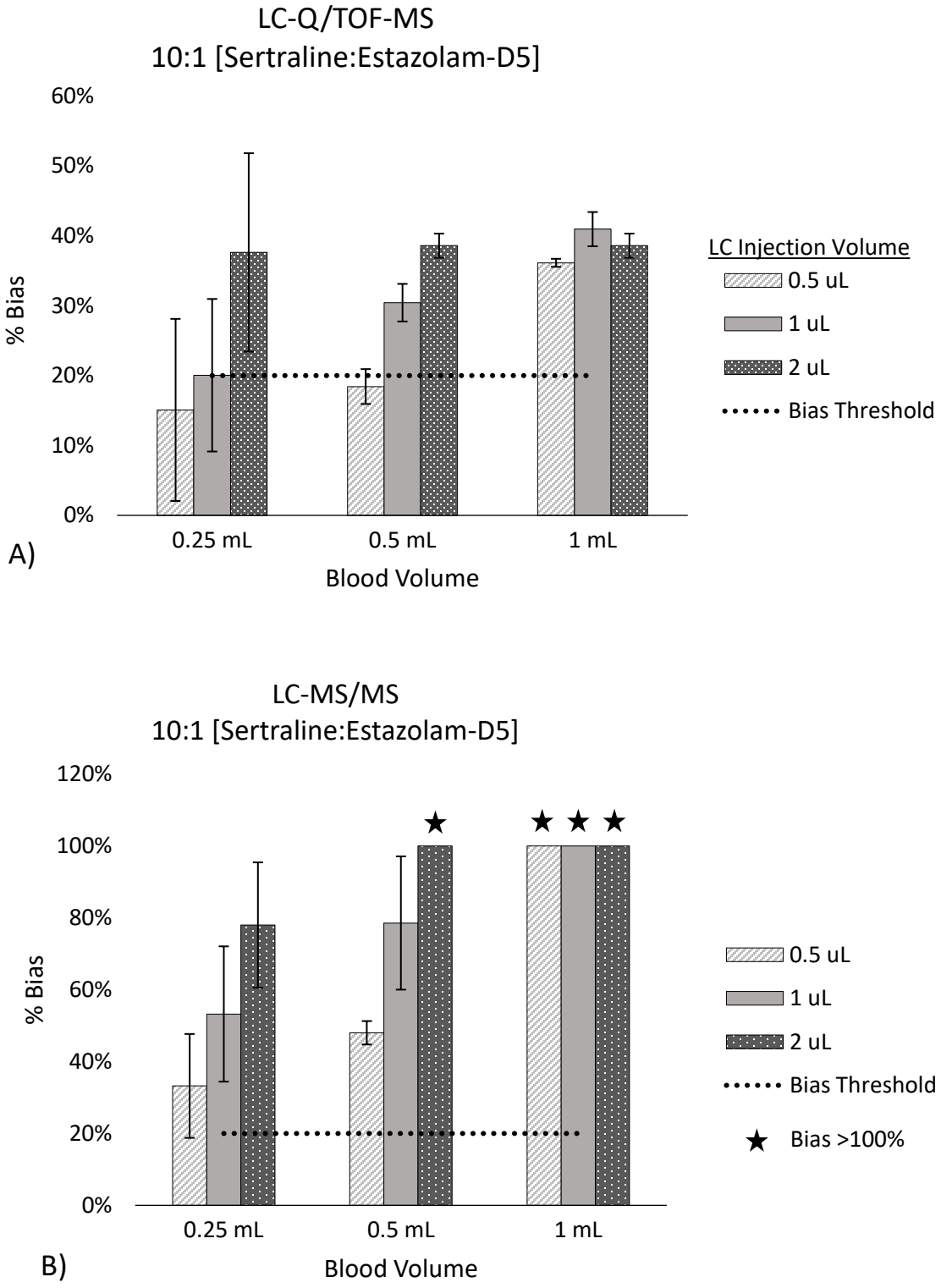


Figure 5.5. Quantitative bias observed in the LC-Q/TOF method (A) and the LC-MS/MS method (B) using various sample and injection volumes when sertraline was present at a 10-fold excess relative to the IS (n=3, mean ± 1SD).

Although a stable isotope labeled internal standard for suvorexant was not commercially available during the time of the initial LC-Q/TOF-MS and LC-MS/MS method development and validation (15, 16), one recently became available. Suvorexant-D6 was added to the LC-Q/TOF targeted data acquisition method, and product ions (**Table 5.2**) were selected for quantitation with a weighted (1/x) quadratic model (2-200 ng/mL) as described earlier (15). Interferences from the same 52 drugs were evaluated once more. As expected, no qualitative or quantitative interferences were identified even at 10-fold and 100-fold excess concentrations relative to the drug (suvorexant). Additionally, the sertraline and propoxyphene interference experiments were repeated at concentration ratios 1:10, 1:1, and 10:1 relative suvorexant-D6 (100 ng/mL), and quantitative bias was <20% at all concentrations (**Figure 5.6**).

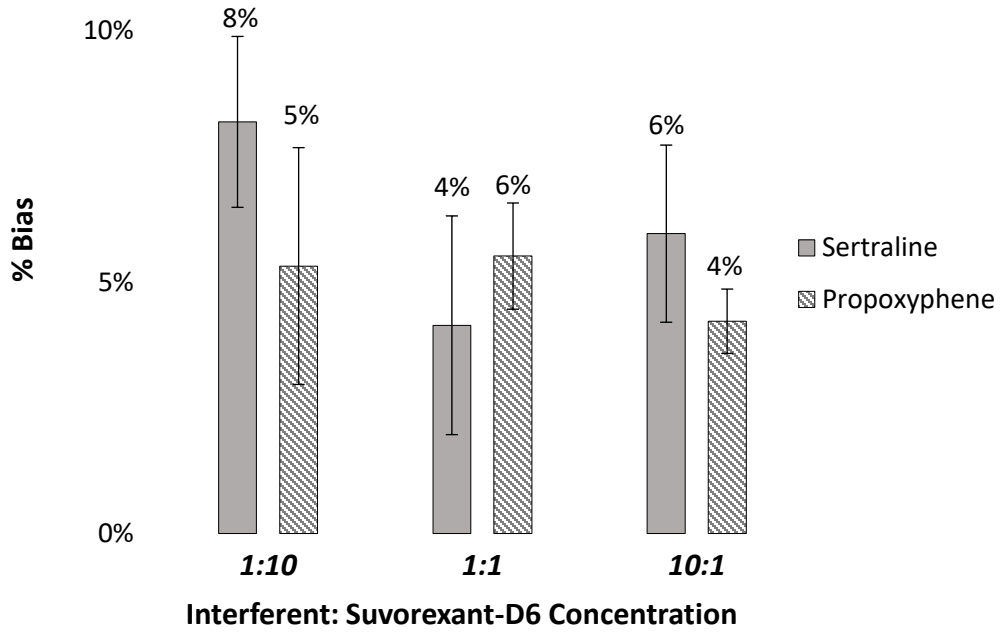


Figure 5.6. Quantitative bias (>20%) observed for suvorexant in the presence of interfering drugs at concentration ratios of 1:10, 1:1 and 10:1 interferent: suvorexant-D6 (n=3, mean ± 1SD).

Dilution Integrity

Dilution integrity studies are required if the quantity (i.e., volume) of specimen used is subject to modification. When performing such studies, the reduced volume of specimen may remain undiluted, or may be diluted with aqueous media or buffer prior to the extraction. In this study, we compared dilution integrity at 1:1 and 1:10 using estazolam-D5 and suvorexant-D6 as in the internal standard. Statistical significance was assessed using a two-tailed student t-test ($\alpha = 0.05$). When dilutions were performed at 1:1 with estazolam-D5 as the IS, no significant differences in concentration were observed between the diluted and undiluted positive control when the dilution was performed using drug-free blood. However, significant differences were observed between the positive controls when the specimen was diluted with buffer, and when the sample volume was halved with no dilution (**Figure 5.7A**). When a 1:10 dilution was performed using estazolam-D5 as the internal standard, concentrations were significantly different using each dilution method (**Figure 5.7B**). The experiment was repeated using suvorexant-D6 as the internal standard, and measured concentrations and bias were not statistically significant for 1:1 dilution using one-way ANOVA ($F(3,12) = 0.30$, $p = 0.83$, $\alpha = 0.05$)) or 10:1 ($F(3,12) = 2.0$, $p = 0.17$, $\alpha = 0.05$) regardless of how the dilution was performed (**Figures 5.7A & 5.7B**).

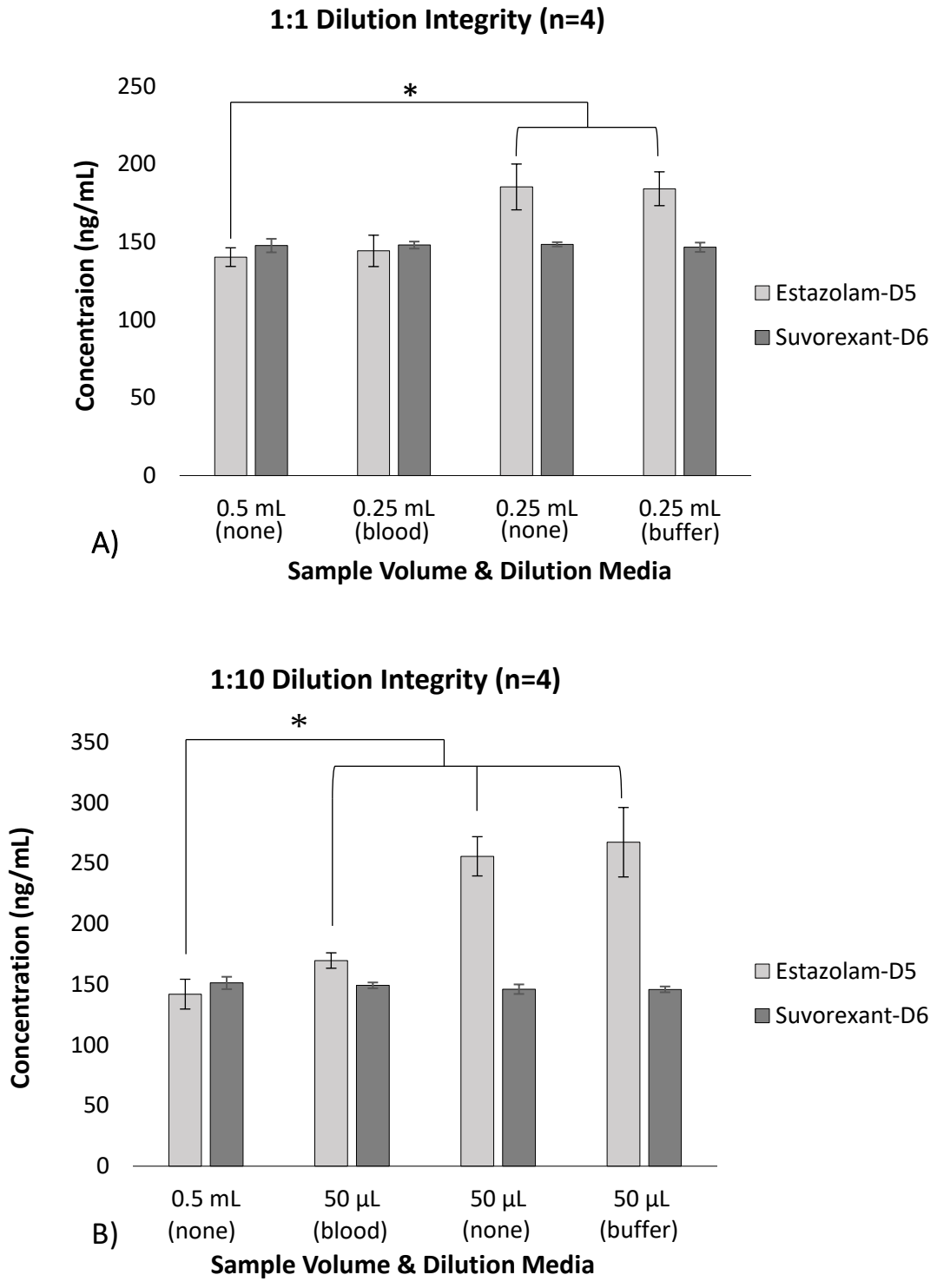


Figure 5.7. Comparison of dilution integrity at 1:1 (A) and 1:10 (B) using blood, buffer or decreased sample volume alone (160 ng/mL suvorexant & 100 ng/mL IS) (n=4, mean ± 1SD).

These observed differences due to the composition of the dilution medium (i.e., none, buffer or drug-free blood) were attributed to the influence of viscosity on extraction efficiency. Despite some structural similarity (diazepine/azepane, triazole and halogenation), extraction efficiencies for estazolam-D5 and suvorexant are not identical. Liquid-liquid extraction efficiencies rely upon the physical mixing and interaction of analyte between the biological matrix and the solvent layer. As such, they are influenced by viscosity. The observed differences between the dilution medium once again highlights the value of isotope labeled internal standards to compensate for analytical factors during extraction.

Conclusion

ESI based LC-MS techniques are undoubtedly one of the most valuable tools in forensic toxicology today. However, additional care is needed during method validation to ensure that analytical methods are free from quantitative bias caused by coeluting substances (matrix, or drug). While much attention is placed on quantitative matrix effects, ion suppression caused by other drugs that may also coelute is often overlooked or not thoroughly investigated. These should be investigated qualitatively and quantitatively during method development so that potential limitations can be identified. Matrix effects and other interferences can have a detrimental impact on the precision and bias of quantitative LC-ESI-MS methods and can ultimately results in decreased sensitivity (4, 5, 10, 21). While endogenous interferences are often discussed in LC-based methods, exogenous interferences can also be present which can include extraction artifacts, buffers, and coeluting drugs (3, 10). There has been a common misconception that high resolution mass spectrometry and tandem mass spectrometry techniques are less susceptible to the

effects of interferences due to their mass selectivity, namely the filtering capabilities in tandem MS and mass accuracy in HRMS (4, 21); but the suppression caused by capacity limited ionization occurs before ions enter the mass spectrometer, making these methods just as susceptible to interferences due to coelution. As demonstrated here, both high resolution and tandem mass spectrometry techniques can suffer from this limitation. If not thoroughly investigated, quantitative analyses might be subject to considerable positive or negative bias. These effects can be mitigated, however.

Stable isotope labeled internal standards can be used to overcome these issues in LC-MS assays (1, 2). Alternative measures are also possible in instances where an SIL-IS in not available, particularly new or emerging drugs. Reducing the number of species present in solvent droplets by decreasing sample (matrix) volume, LC injection volume, or source conditions can help. However, these solutions are compound-dependent and must be investigated on an individual basis. Regardless, sources of ion suppression beyond just those attributed to matrix effects should be thoroughly evaluated qualitatively and quantitatively in a forensic toxicology setting.

References

1. Furey, A., Moriarty, M., Bane, V., Kinsella, B. and Lehane, M. (2013). Ion suppression; A critical review on causes, evaluation, prevention and applications. *Talanta*. **115**, 104-122.
2. Peters, F.T. and Remane, D. (2012) Aspects of matrix effects in applications of liquid chromatography–mass spectrometry to forensic and clinical toxicology—a review. *Analytical and Bioanalytical Chemistry*. **403**, 2155-2172.
3. Larger, P.J., Breda, M., Fraier, D., Hughes, H. and James, C.A. (2005) Ion-suppression effects in liquid chromatography–tandem mass spectrometry due to a formulation agent, a case study in drug discovery bioanalysis. *Journal of Pharmaceutical and Biomedical Analysis*. **39**, 206-216.
4. Matuszewski, B.K., Constanzer, M.L. and Chavez-Eng, C.M. (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*. **75**, 3019-3030.
5. Taylor, P.J. (2005) Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry*. **38**, 328-334.
6. Kebarle, P. and Tang, L. (1993) From ions in solution to ions in the gas phase - the mechanism of electrospray mass spectrometry. *Analytical Chemistry*. **65**, 972A-986A.
7. Janusson, E., Hesketh, A.V., Bamford, K.L., Hatlelid, K., Higgins, R. and McIndoe, J.S. (2015) Spatial effects on electrospray ionization response. *International Journal of Mass Spectrometry*. **388**, 1-8.

8. Ismaiel, O.A., Halquist, M.S., Elmamly, M.Y., Shalaby, A. and Thomas Karnes, H. (2008) Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations. *Journal of Chromatography B*. **875**, 333-343.
9. Gosetti, F., Mazzucco, E., Zampieri, D. and Gennaro, M.C. (2010) Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*. **1217**, 3929-3937.
10. Chambers, E., Wagrowski-Diehl, D.M., Lu, Z. and Mazzeo, J.R. (2007) Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography B.* **852**, 22-34.
11. Ghosh, C., Shinde, C.P. and Chakraborty, B.S. (2012) Influence of ionization source design on matrix effects during LC–ESI-MS/MS analysis. *Journal of Chromatography B*. **893-894**, 193-200.
12. Jian, W., Edom, R.W., Xu, Y., Gallagher, J. and Weng, N. (2010) Potential bias and mitigations when using stable isotope labeled parent drug as internal standard for LC–MS/MS quantitation of metabolites. *Journal of Chromatography B*. **878**, 3267-3276.
13. Mortier, K.A., Verstraete, A.G., Zhang, G.-F. and Lambert, W.E. (2004) Enhanced method performance due to a shorter chromatographic run-time in a liquid chromatography–tandem mass spectrometry assay for paclitaxel. *Journal of Chromatography A*. **1041**, 235-238.

14. Berg, T. and Strand, D.H. (2011) ^{13}C labelled internal standards—A solution to minimize ion suppression effects in liquid chromatography–tandem mass spectrometry analyses of drugs in biological samples? *Journal of Chromatography A*. **1218**, 9366-9374.
15. Skillman, B. and Kerrigan, S. (2018) Quantification of suvorexant in blood using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry. *Journal of Chromatography B*. **1091**, 87-95.
16. Skillman, B. and Kerrigan, S. (2019) Identification of Suvorexant in Blood Using LC-MS/MS: Important Considerations for Matrix Effects and Quantitative Interferences in Targeted Assays. *Journal of Analytical Toxicology*. DOI 10.1093/jat/bkz083.
17. U.S. Food and Drug Administration (2013) Suvorexant Tablets, Insomnia Indication, Peripheral & Central Nervous System Drugs Advisory Committee Meeting. Whitehouse Station, NJ, Federal Drug Administration.
18. U.S. Drug Enforcement Administration (2014) Schedules of Controlled Substances: Placement of Suvorexant into Schedule IV. Final Rule, Federal Register. **79**, 51243-51247.
19. Scientific Working Group for Forensic Toxicologists (2013) Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. *Journal of Analytical Toxicology*. **37**, 452-474.
20. Villagrasa, M., Guillamón, M., Eljarrat, E. and Barceló, D. (2007) Matrix effect in liquid chromatography–electrospray ionization mass spectrometry analysis of

benzoxazinoid derivatives in plant material. *Journal of Chromatography A*. **1157**, 108-114.

21. Trufelli, H., Palma, P., Famiglini, G. and Cappiello, A. (2011) An overview of matrix effects in liquid chromatography-mass spectrometry. *Mass Spectrometry Reviews*. **30**, 491-509.

CHAPTER VI

CYP450-MEDIATED METABOLISM OF SUVOREXANT AND INVESTIGATION OF METABOLITES IN AUTHENTIC CASE SPECIMENS

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

Abstract

Suvorexant (Belsomra®) is a sedative hypnotic that was approved for use in 2015. It has a novel mechanism of action and was the first dual orexin receptor antagonist (DORA) to be approved for the treatment of sleep disorders. Sedative hypnotics often feature prominently in forensic investigations such as impaired driving and drug-facilitated sexual assault (DFSA) cases. As such, suvorexant is a drug of interest and its identification in forensic toxicology investigations is of significance. However, limited studies have been published to date and the disposition or importance of its metabolites has been largely uninvestigated. Only one study to date has described the metabolism of suvorexant. In this report, we investigate the enzymes responsible for metabolism and explore the prevalence of metabolites in blood from a series of thirteen forensic investigations. Recombinant cytochrome P450 enzymes (rCYPs) were used to generate phase I metabolites for suvorexant *in vitro* and metabolites were identified using liquid chromatography-quadrupole/time-of-flight-mass spectrometry (LC-Q/TOF-MS). Four rCYP isoenzymes (3A4, 2C19, 2D6, and 2C9) were found to contribute to suvorexant metabolism. The only metabolite identified in blood or plasma arose from hydroxylation of the benzyl triazole moiety (M9). This metabolite was identified in seventeen blood and plasma specimens from twelve medicolegal death investigations and one impaired driving investigation. In the absence of a commercially available reference material, the metabolite was confirmed using rCYP-generated *in vitro* controls using high resolution mass spectrometry.

Keywords: Suvorexant, Metabolism, CYP450, Blood, Plasma, LC-Q/TOF-MS

CYP450-MEDIATED METABOLISM OF SUVOREXANT AND INVESTIGATION OF METABOLITES IN AUTHENTIC CASE SPECIMENS

Introduction

Suvorexant is novel dual orexin receptor antagonist (DORA) that is marketed under the tradename Belsomra® and is used for the treatment of insomnia. Orexin antagonism is a novel approach for treating insomnia. Orexin neurons were only discovered in the late 1990s by two independent research groups and have been implicated in the sleep to wake cycle, as production of orexin results in decreased activity in the sleep-promoting parts of the brain (1-3). In postmortem studies of individuals that suffered from narcolepsy it has been discovered that there was a lack of orexin-producing neurons in the lateral hypothalamus, further suggesting that the orexin signaling plays a large role in regulating wakefulness (3-6). With this discovery, dual orexin receptor antagonists have been developed to mimic these effects and to provide alternative therapeutic options for insomnia treatment. Suvorexant acts by inhibiting orexin A and B in the lateral hypothalamus to produce suppression of the wake cycle, thereby inducing sleep in its users. Unlike other traditional sedative hypnotics prescribed for insomnia, suvorexant has no effect on γ-aminobutryic acid (GABA) activity and is thought to have a lower abuse potential. Moreover, DORAs are a promising approach due to the pharmacokinetics of these compounds, which aim to restrict their effects to the duration of sleep with minimal carryover or “hangover” effects into the following day after bedtime use (2).

Suvorexant became commercially available in 2015 and is currently placed under Schedule IV of the Controlled Substances Act (CSA) (7). Suvorexant is currently available in the United States, Japan, and Canada (8-10). Following a 10 mg oral dose, peak plasma

concentrations are ~198 ng/mL which are reached within 2 hours, and steady state plasma concentrations can be reached within three days of daily suvorexant administration (11, 12). Suvorexant is recommended to be administered within 30 minutes of going to sleep and at least 7 hours before the anticipated time of wakening (13, 14). The mean oral bioavailability of suvorexant is ~82% and it is extensively protein bound (99.5%) to human serum albumin and α-1-acid glycoprotein (14, 15). The drug has a relatively long half-life, is highly lipophilic, and has volume of distribution of 0.5-0.9 L/kg (9, 16, 17).

Suvorexant is a drug of forensic interest due to its relatively long half-life (~12 hours on average) and its classification as a sedative hypnotic. In the prescribing information, administration of the high dose (20 mg) is discouraged prior to driving and other next-day activities that require alertness (15). Suvorexant has the potential to appear in impaired driving investigations due to its adverse side effects, or even in cases of drug-facilitated sexual assault (DFSA). However, few methods have been developed for its analysis in biofluids of forensic interest. Methods for the detection of suvorexant in urine, blood, plasma, and tissues have been developed for use with gas chromatography-mass spectrometry (GC-MS), liquid chromatography tandem mass spectrometry (LC-MS/MS), and liquid chromatography-quadrupole/time-of-flight-mass spectrometry (LC-Q/TOF-MS) (18-26). Only one of these methods has examined suvorexant in case specimens from three forensic autopsy cases (24). Therefore, little is known about its role in human performance toxicology or in forensic investigations, and its potential for postmortem redistribution should be evaluated with more cases studies. Moreover, only one study has evaluated the *in vivo* and *in vitro* metabolism of suvorexant, and no studies have been

published to date describing the analysis of suvorexant metabolites in forensic casework (11).

Ciu et al. is the only research group to date that has studied the *in vivo* and *in vitro* metabolism of suvorexant (11). The metabolism of suvorexant was investigated in a clinical dosing study with the use of healthy volunteers. A radiolabeled dose (50 mg) of suvorexant ([^{14}C]-suvorexant) was orally administered to individuals, and elimination was monitored over 14 days. Suvorexant was excreted principally via the feces (66%), with only 23% of the drug eliminated in the urine. Identification of potential metabolites was performed using high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR). In plasma, suvorexant (30%) and a hydroxylated metabolite, M9 (37%) were the principal compounds detected. The chemical structures for these two species can be found in **Figure 6.1**. Further oxidation of M9 produced the carboxylic acid derivative (M4), which was the principal metabolite in urine, together with its glucuronide. M4 was also the major metabolite in feces, accounting for approximately 17% of a dose. In addition to hydroxylation of the benzyl alcohol (M9), hydroxylation also occurred at the chlorobenzoxazole (M8) and methyldiazapane ring (M10a), followed by glucuronidation. Dechlorinated species were also reported and mechanisms for their formation were postulated (11). The metabolites were extensively protein-bound, similar to the parent compound.

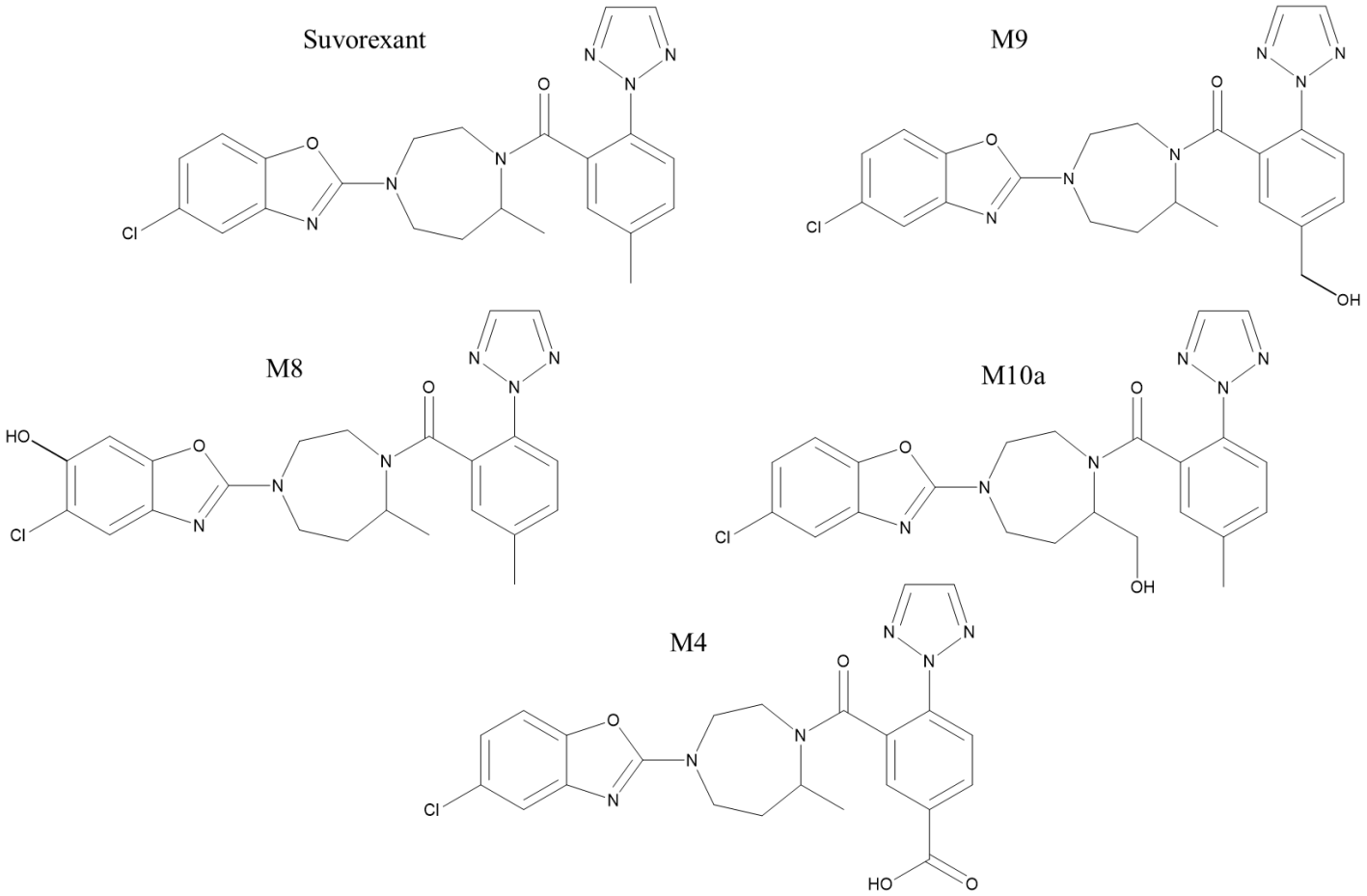


Figure 6.1. Chemical structures of suvorexant and selected metabolites.

The *in vitro* characterization of suvorexant metabolism was performed using human liver microsomes (HLMs) and selected recombinant CYP450 enzyme systems (3A4, 3A5 and 2C19). Immunoinhibition studies were performed using specific anti-CYP monoclonal antibodies (anti-CYP 1A2, 3A4/5, 2C8/9/19 and 2D6). It was concluded that CYP 3A4 was the principal enzyme involved in metabolism, with only minor contributions from CYP 2C19. No CYP 1A2, 2C8 or 2D6 activity was observed. Data showed that suvorexant was well absorbed (>90%) and extensively metabolized. As a result, suvorexant metabolites account for a large fraction of circulating species present following oral administration of the drug (11). Although none of the metabolites are believed to be pharmacologically active, none are commercially available. This presents a challenge for forensic analysis and would preclude quantitative analysis if parent/metabolite ratios were of interest.

HRMS techniques are a powerful bioanalytical tool and have gained popularity in identifying metabolites using *in vivo* and *in vitro* approaches. Traditionally, NMR analysis was used for structural elucidation of metabolite in metabolomic studies, but HRMS has become increasingly used due to high sensitivity and structural elucidation capabilities using accurate mass and MS/MS fragmentation patterns (27, 28). In this study, metabolism of suvorexant was investigated *in vitro* for the purpose of identifying suvorexant metabolites in specimens from thirteen forensic investigations. To produce metabolite controls for the purpose of identifying these compounds in human blood, serum/plasma, and vitreous humor, recombinant CYP isoenzyme incubations were performed *in vitro* using eight isoenzymes. Following confirmation of enzyme activity and metabolite production, these isoenzyme incubations were used as positive controls to qualitatively

evaluate metabolites in these specimens. The potential utility of suvorexant metabolite identification in forensic investigations is discussed.

Materials and Methods

Chemicals and Reagents

Suvorexant was obtained from Cayman Chemical (Ann Arbor, MI, USA) in powdered form and was prepared in methanol at 1 mg/mL. Suvorexant-D6 (100 µg/mL), estazolam-D5 (100 µg/mL), ketoconazole (2 mg/mL) and fluvoxamine (1 mg/mL) were obtained from Cerilliant Corp. (Round Rock, TX, USA) in methanol. Acetonitrile (Optima, LC/MS grade) and formic acid (Optima, LC/MS grade) were obtained from Fisher Scientific (Pittsburg, PA, USA). Methanol (LC/MS grade) was purchased from J.T. Baker (Center Valley, MA, USA). A Millipore Direct-Q® UV Water Purification System (Billerica, MA) was used for the in-house purification of deionized water. Sodium acetate and glacial acetic acid (used to prepare sodium acetate buffer (pH 3.6, 0.4 M)), were obtained from Mallinckrodt Chemicals (St. Louis, MO, USA). *N*-butyl chloride (1-chlorobutane, 99+% pure) was purchased from Fisher Scientific (Pittsburg, PA, USA) and potassium phosphate (mono and dibasic) were obtained from VWR (Radnor, PA, USA). Whole bovine blood for controls and calibrators was obtained from QuadFive (Ryegate, MT, USA). Sodium fluoride (1% w/v) and potassium oxalate (0.2% w/v) were used as preservative and anticoagulant. Recombinant human cytochrome P450 (rCYP) isoenzymes expressed in *Escherichia coli* (bactosomes) and control bactosomes were purchased from Sekisui Xenotech, LLC (Kansas City, KS, USA). Reduced nicotinamide adenosine di-phosphate (NADPH) regenerating systems were obtained from Corning® Gentest™ (Glendale, AZ, USA). NADPH system solution A consisted of 40 U/mL glucose-6-

phosphate dehydrogenase in 5 mM sodium citrate and NADPH solution B consisted of 26 mM $NADP^+$, 66 mM glucose-6-phospate, and 66 mM magnesium chloride in aqueous solution.

Instrumentation

An Agilent 1290 Infinity Binary LC system coupled to an Agilent 6530 Accurate Mass Quadrupole Time-of-Flight LC/MS (Santa Clara, CA) was used for identification and analysis of metabolites. Mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. The LC was equipped with an Agilent InfinityLab Poroshell 120 EC-C18 column (2.1 x 100 mm x 2.7 µm) and Poroshell EC-C18 guard column (2.1 x 5 mm x 2.7 µm) that were maintained at 35°C. Separation was achieved using the following gradient: Begin 40% B, hold 40% B (3 min), increase 40-80% B (1 min), hold 80% B (2 min), decrease 80-40% B (1 min), followed by post-equilibration (2 min). A 6-second needle wash was employed to prevent carryover. Electrospray ionization was used in positive mode under the following conditions: 300°C drying gas temperature (13 L/min), 350°C sheath gas temperature (12 L/min), 45 psi nebulizer pressure, 150 V fragmentor voltage, 2000 V nozzle voltage, and 3000 V capillary voltage. Collision induced dissociation was evaluated at 10, 30, and 50 eV. Auto MS/MS (full scan) data acquisition was used for a mass range of 40-1000 amu using medium isolation widths (~4 amu). The MS scan rate was 3 spectra/sec while the MS/MS scan rate was 8 spectra/sec. Previous literature-reported metabolite exact masses were calculated using MassHunter mass calculator and masses were added to a preferred list in the auto MS/MS method. MassHunter Qualitative Analysis software was used for data analysis. Phase I metabolites were identified using mass accuracy (± 5 ppm) and MS/MS fragmentation patterns.

Recombinant CYP Incubations

Eight rCYP isoenzymes were evaluated for the production of suvorexant metabolites *in vitro* as follows: CYPs 3A4, 2C19, 2D6, 2C9, 2C8, 2C18, 2B6, and 1A2. Each isoenzyme was incubated individually to evaluate its contribution to suvorexant metabolism. In accordance with manufacturer recommendations and previously published studies, 50 µM of suvorexant and 50 pmol/mL rCYP isoenzyme was used for incubations at 37°C (29). The total reaction volume was 0.5 mL which also included 100 mM potassium phosphate buffer (pH 7.4), 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM magnesium citrate, and 0.4 U/mL glucose-6-phosphate dehydrogenase. Time points of 0, 30, 60, 120, 180, and 240 minutes were evaluated for metabolite formation. Reactions were quenched by taking an incubation mixture aliquot of 25 µL and adding to an equal volume of stop solution consisting of 5 µM internal standard (suvorexant-D6) and 0.1% formic acid in acetonitrile. The solution was centrifuged at 4°C for 3 minutes at 10,000 x g and 12.5 µL of the resulting supernatant was diluted (1:1) with mobile phases A/B (50/50). Control incubations which contained control bactosomes (no CYP insertion) were prepared similarly to rCYP incubations and blanks (no drug) for each rCYP were prepared by replacing suvorexant solution with phosphate buffer. An LC injection volume of 2 µL was used for sample introduction and analysis.

Inhibition

Inhibition was studied using known chemical inhibitors for rCYPs 3A4, 2C19, 2D6, and 2C9. For rCYP 3A4, 2C19, and 2C9 inhibition ketoconazole (40 µM) was used, and for rCYP 2D6 inhibition fluvoxamine (40 µM) was used. Incubations were performed for each of the four isoenzymes with and without the presence of inhibitor in triplicate. The

previously described incubation procedure was utilized with the addition of ketoconazole or fluvoxamine to the set of inhibited samples. Inhibition was studied following a 60-minute incubation, in which aliquots were removed, quenched, centrifuged, and diluted for LC-Q/TOF-MS analysis.

Identification of Metabolites

Metabolite formation was identified by monitoring changes in relative suvorexant response (normalized to the response of suvorexant-D6) over time (0-240 min). A preferred list of suspected metabolites was used to improve the quality of MS/MS spectra. CID energies of 10, 30 and 50 eV were used, and structural assignments were made where possible based upon mass accuracy.

Qualitative Identification in Authentic Specimens

Suvorexant was previously quantitated in authentic blood and serum/plasma specimens from thirteen forensic investigations using a published, validated analytical method (25). Briefly, 0.5 mL of specimen was extracted using a simple acidic/neutral liquid-liquid extraction (LLE) with 1 mL sodium acetate buffer (pH 3.6, 0.4 M) and 2.5 mL *N*-butyl chloride, and estazolam-D5 (100 ng/mL) was used as the internal standard. Samples were rotated for 5 min and centrifuged for 5 min at 1734 x *g* (3000 rpm). Organic solvent was transferred to conical tubes and dried under nitrogen stream (50°C). Samples were reconstituted in 50:50 mobile phase A/B, transferred to LC vials, and 2 µL were injected into the instrument. The previous quantitative method resulted in an LOD and LOQ of 0.5 ng/mL and calibration range of 2-200 ng/mL using a weighted (1/x) quadratic calibration model. In this study metabolites were qualitatively identified in the same antemortem and postmortem authentic specimens following fortification with estazolam-

D5 and suvorexant-D6 internal standards (100 ng/mL) and extraction with the described LLE. Whole bovine blood was used for negative (100 ng/mL ISs) and positive (100 ng/mL ISs, 100 ng/mL suvorexant) controls. In the absence of commercially available suvorexant metabolite reference materials or standards, positive metabolite controls were produced *in vitro* with rCYP incubations. Metabolite data was acquired for samples using auto MS/MS as described, and metabolites were confirmed using retention time matching to positive rCYP incubations, mass accuracy (±5 ppm), and mass spectral fragmentation patterns.

Results and Discussion

Identification of Phase I Metabolites

A total of three suvorexant metabolites were identified which were all produced by hydroxylation of the parent compound (m/z 467) and eluted at 3.03, 3.18, and 4.37 minutes. The chromatographic separation of suvorexant, suvorexant-D6, and the hydroxysuvorexant isomers is depicted in **Figure 6.2**. and MS/MS spectra are shown in **Figure 6.3**. Chemical formula, exact mass, accurate mass, and mass errors are summarized in **Table 6.1**. All mass errors for metabolite precursor ions were within ± 5 ppm. Suvorexant produces a prominent m/z 186 ion, consistent with the benzyltriazole fragment ($C_{10}H_8N_3^+$). Hydroxysuvorexant isomer 2 was readily identified as the M9 metabolite due to the m/z 202 ion, consistent with the benzyl alcohol fragment. Two other minor metabolites (isomers 1 and 3) were identified. The prominence of the m/z 186 ion indicated that hydroxylation occurred elsewhere on the molecule, such as the chlorobenzoxazole group (M8) or methyldiazepane ring (M10a) as suggested by Ciu et al. (11). Structural assignments for suvorexant and metabolites product ions with their respective mass errors at each collection energy used

(10, 30, 50 eV) are shown in **Table 6.2**. Due to the absence of diagnostic fragment ions (even at elevated CID voltages), further differentiation was not possible.

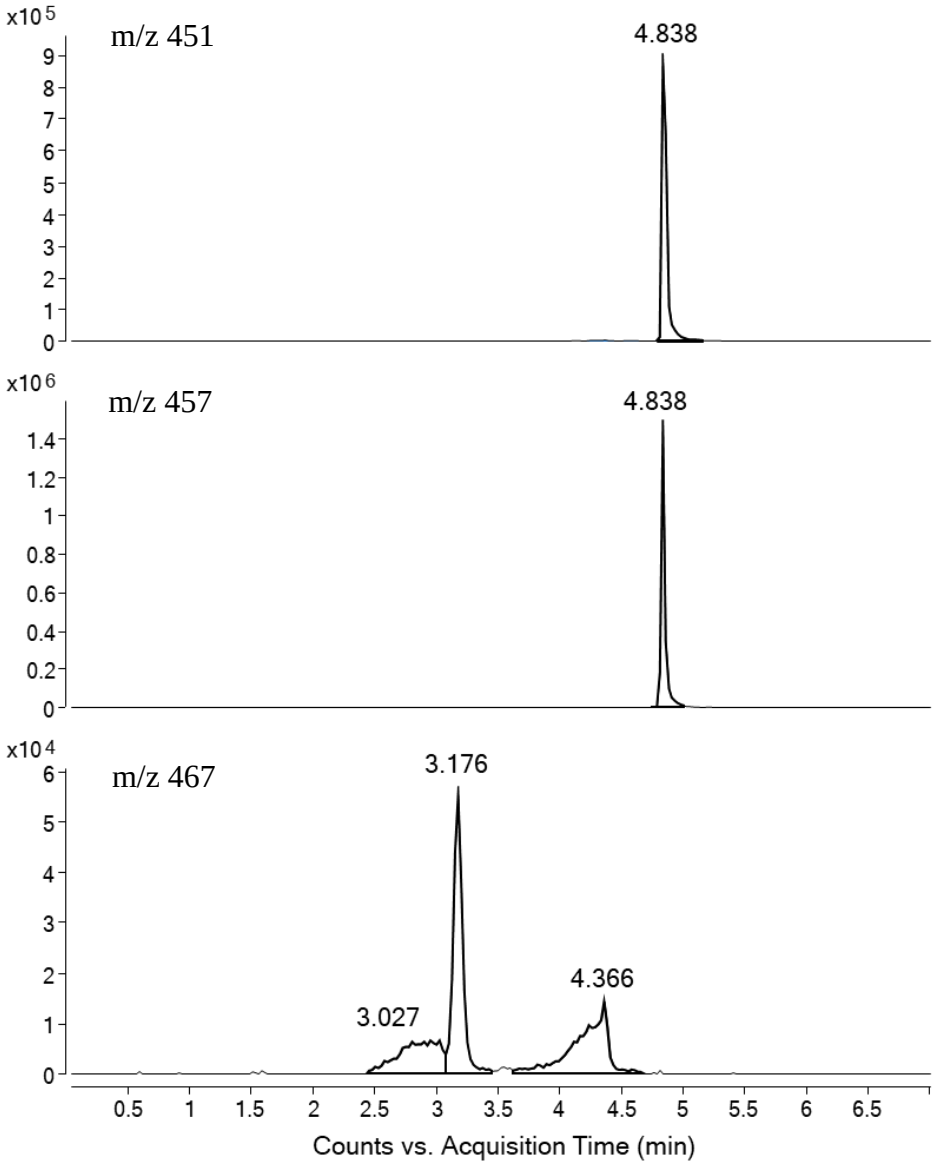


Figure 6.2. Extracted ion chromatograms for suvorexant (4.838 min; m/z 451), suvorexant-D6 (4.838 min; m/z 457), and hydroxylated species (3.027, 3.176, 4.366 min; m/z 467).

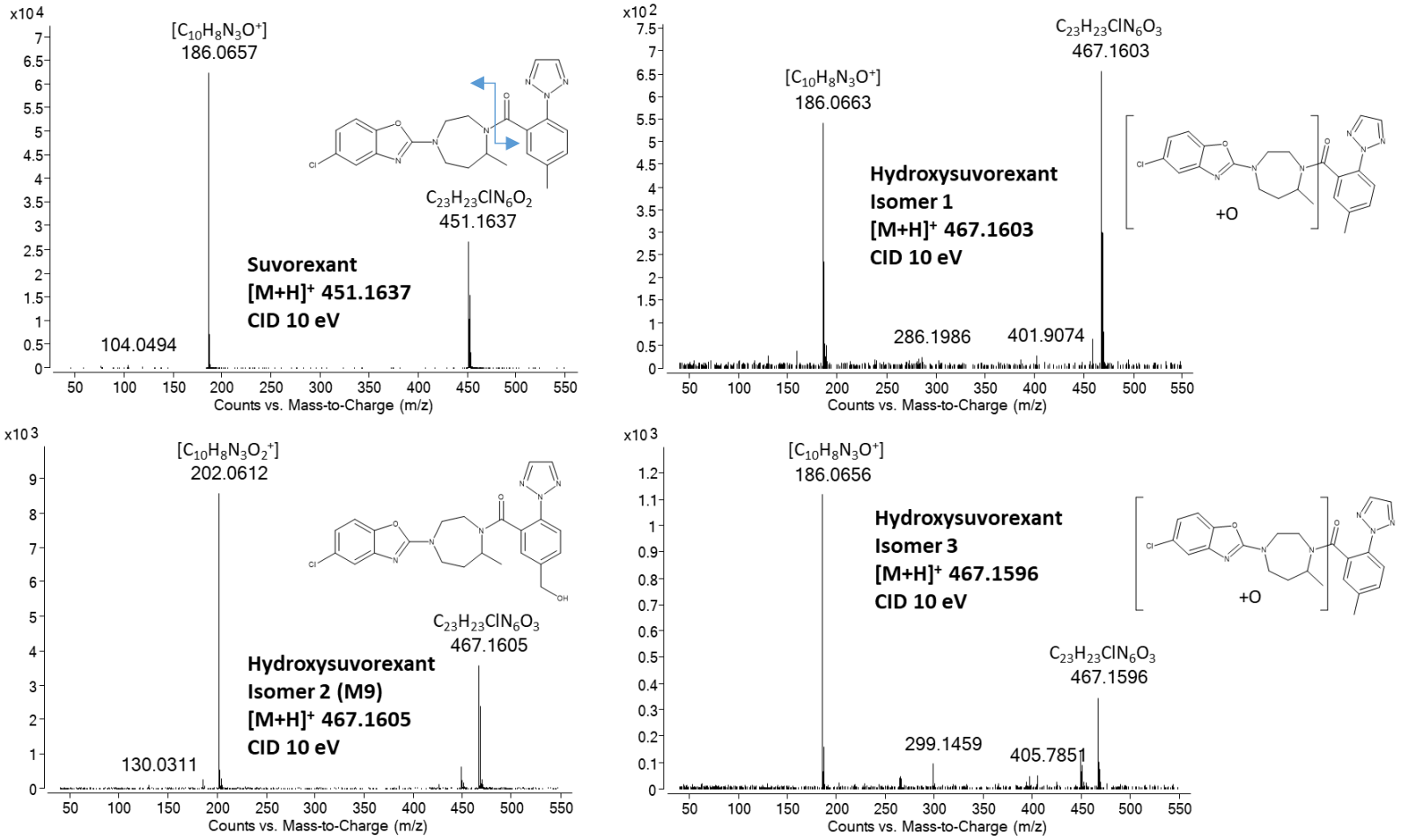


Figure 6.3. MS/MS spectra of suvorexant and three hydroxylated metabolites.

Table 6.1. Retention time, chemical formulas, exact mass, accurate mass, and mass error for suvorexant and its hydroxylated metabolites.

Compound	Retention Time (min)	Chemical Formula	Exact Mass $(M+H)^+$	Accurate Mass $(M+H)^+$	Mass error (ppm)
Suvorexant	4.84	$C_{23}H_{23}ClN_6O_2$	451.1644	451.1637	-1.55
Hydroxysuvorexant isomer 1	3.03	$C_{23}H_{23}ClN_6O_3$	467.1593	467.1603	+2.14
Hydroxysuvorexant isomer 2 (M9)	3.18	$C_{23}H_{23}ClN_6O_3$	467.1593	467.1605	+2.57
Hydroxysuvorexant isomer 3	4.37	$C_{23}H_{23}ClN_6O_3$	467.1593	467.1596	+0.64

Table 6.2. Chemical formulas, exact mass, accurate mass, and mass error for product ions of hydroxylated metabolite isomers at different collision energies (10, 30 50 eV).

Compound	CID Voltage	Chemical Formula	Exact Mass $(M+H)^+$	Accurate Mass $(M+H)^+$	Mass error (ppm)
Suvorexant	10	$[C_{10}H_8N_3O^+]$	186.0662	186.0657	-2.69
	30	$[C_{10}H_8N_3O^+]$	186.0662	186.0660	-1.07
	50	$[C_{10}H_8N_3O^+]$	186.0662	186.0658	-2.15
Hydroxysuvorexant isomer 1	10	$[C_{10}H_8N_3O^+]$	186.0662	186.0663	+0.54
	30	$[C_{10}H_8N_3O^+]$	186.0662	186.0658	-2.15
	50	$[C_{10}H_8N_3O^+]$	186.0662	186.0665	+1.61
Hydroxysuvorexant isomer 2 (M9)	10	$[C_{10}H_8N_3O_2^+]$	202.0611	202.0612	+0.49
	30	$[C_{10}H_8N_3O_2^+]$	202.0611	202.0607	-1.98
	50	$[C_{10}H_8N_3O_2^+]$	202.0611	202.0613	+0.99
Hydroxysuvorexant isomer 3	10	$[C_{10}H_8N_3O^+]$	186.0662	186.0656	-3.22
	30	$[C_{10}H_8N_3O^+]$	186.0662	186.0654	-4.30
	50	$[C_{10}H_8N_3O^+]$	186.0662	186.0669	+3.76

rCYP Activity

Using eight different rCYP isoenzymes evaluated, suvorexant metabolites were identified using rCYP 3A4, 2C19, 2D6 and 2C9. The abundance of each metabolite was measured relative to the internal standard (suvorexand-D6). Hydroxysuvorexant isomer 1

was attributed to rCYPs 3A4 and 2C19, while isomer 3 was produced in the presence of rCYPs 3A4 and 2C9. The M9 metabolite, was produced by four isoforms: rCYP 2D6, 3A4, 2C9 and 2C19. Metabolism was not observed for these species in the control isoenzyme incubations or in the blank samples (free from suvorexant).

Inhibition studies were performed to confirm the results using 60-minute incubations. Inhibited and uninhibited reactions were performed in parallel, each in triplicate. The RPAs of suspected metabolites and suvorexant-D6 internal standard were compared for inhibited and uninhibited samples (**Figure 6.4**). Significant inhibition was observed for all isoforms (42-100%) (**Table 6.3**). While the involvement of rCYP 3A4 for the production of hydroxylated isomers 1 and 3 was consistent with Cui et al., activity was also observed using rCYPs 2C9 and 2C19. Notably, Cui et al. did not evaluate rCYP 2C9 in their study. Interestingly they concluded that CYP 2D6 was not involved in the metabolism of suvorexant. In contrast, the M9 metabolite was produced in the greatest abundance by rCYP 2D6 in our study (**Figure 6.4**) and its activity was confirmed in the inhibition study. While this study cannot attest to the *in vivo* formation rates or enzyme kinetics of these metabolites, it does confirm the activity of isoenzymes that were not previously identified.

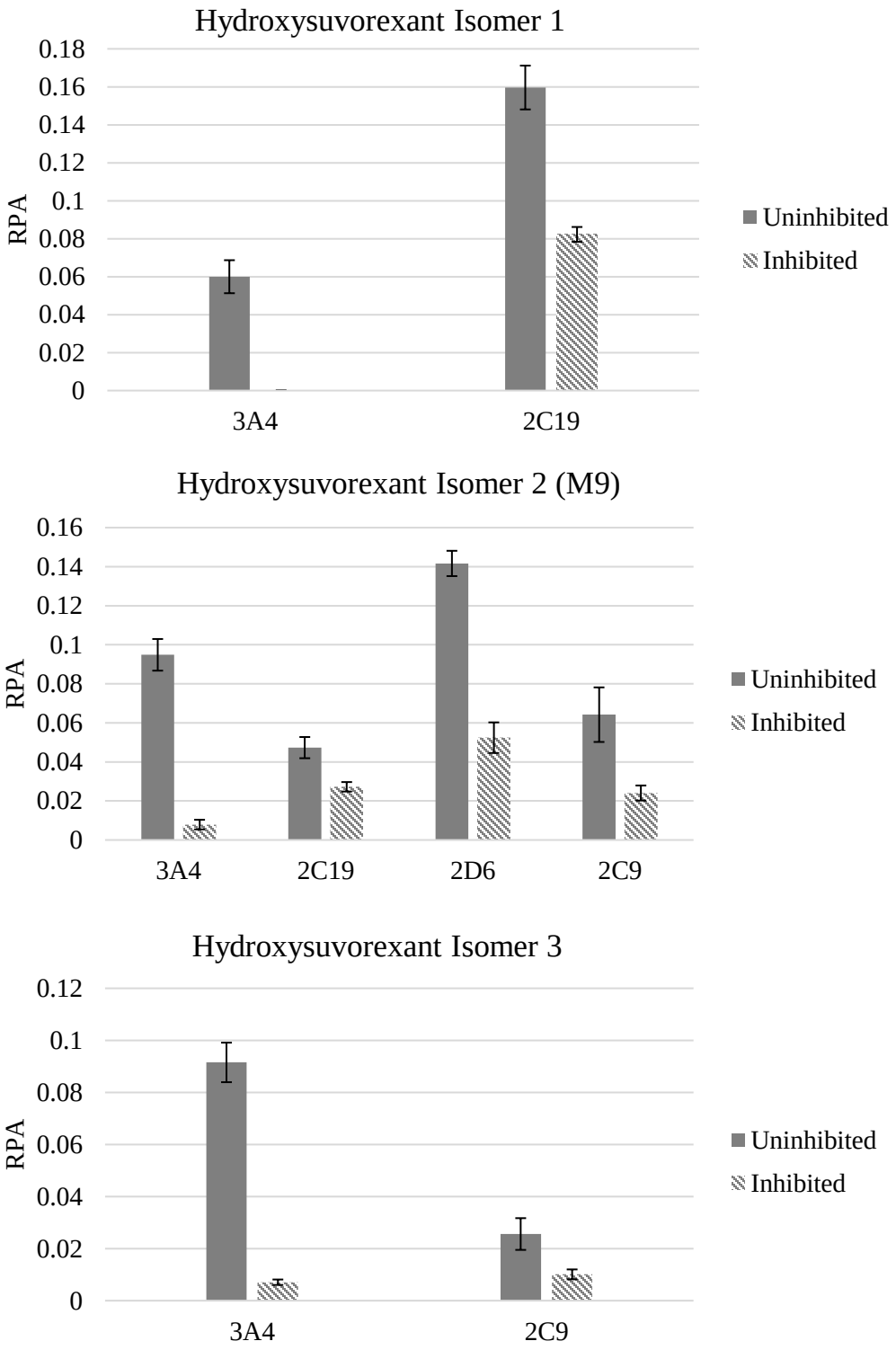


Figure 6.4. Inhibition of hydroxysuvorexant isomer production using rCYPs 3A4, 2C19, 2D6, and 2C9 at 60 min, which is expressed as relative response to the internal standard (suvorexant-D6). Error bars represent ± 1 SD (n=3).

Table 6.3. Percent inhibition for hydroxylated suvorexant metabolites (n=3).

Metabolite	rCYP	% Inhibition (Mean ± SD)
Hydroxysuvorexant isomer 1	3A4 2C19	100 ± 0 48 ± 3
Hydroxysuvorexant isomer 2	3A4 C19 2D6 2C9	92 ± 2 42 ± 9 63 ± 7 62 ± 2
Hydroxysuvorexant isomer 3	3A4 2C9	92 ± 1 60 ± 2

Identification of Metabolites in Authentic Case Specimens

Suvorexant quantitation was previously performed for authentic blood, serum/plasma, and vitreous fluid specimens using a previously validated method and the presence of suvorexant was confirmed. The parent analyte was present in concentrations ranging from 3-42 ng/mL and only vitreous fluid (n=1) could not be reliably quantitated due to extremely low suvorexant concentrations below detection capabilities (LOD=0.5 ng/mL). A total of eighteen specimens were analyzed involving twelve medicolegal death investigations and one impaired driving investigation. The presence of other drugs in the specimens, or the role of suvorexant in the investigation was not known. Since suvorexant metabolite reference materials are not commercially available, positive metabolite controls were produced *in vitro* with rCYP incubations to identify potential suvorexant metabolites in these case specimens. Data was acquired using the auto MS/MS (full scan) method described in this study following LLE of forensic specimens. Negative and positive blood controls were analyzed to ensure precursor ions were not a result of endogenous interfering species. Metabolites were confirmed using retention time matching to positive rCYP incubations, mass accuracy (±5 ppm), and mass spectral fragmentation patterns. Of the thirteen forensic investigations, hydroxysuvorexant (M9) was identified in all case samples with the exception of vitreous humor (**Table 6.4**). The inability to detect metabolites for

suvorexant in this sample is not surprising given the absence of parent drug in this matrix and the low concentration in blood. No other metabolites were detected in the case specimens. A representative extract containing 13.2 ng/mL of suvorexant and M9 metabolite is shown in **Figure 6.5**. All M9 identifications were within ± 5 ppm for mass accuracy, eluted at the correct retention time, and contained matching MS/MS spectra to the control.

Table 6.4. Concentrations of suvorexant in forensic investigations and subsequent identification of M9 metabolite.

Sample #	Case Type	Matrix	Result (ng/mL)	M9 Retention Time (min)	Precursor mass error (ppm)	Product mass error (ppm)
1	DUID	AM Blood	3.2	3.16	1.93	-3.96
2	MDI	Blood (P)	3.5	3.16	3.00	0.00
		Blood (C)	7	3.16	2.35	-1.98
		Vitreous humor	ND	--	--	--
3	MDI	Blood (P)	16.8	3.17	4.07	-3.96
		Blood (C)	36.5	3.15	-0.43	-4.95
4	MDI	AM Blood	21	3.15	4.50	-4.95
		AM Serum/Plasma	3.6	3.15	0.86	-3.46
5	MDI	Blood (P)	36.8	3.15	-1.93	-1.48
6	MDI	Blood (C)	13.2	3.17	-0.64	-2.97
7	MDI	Blood (P)	40.5	3.17	-3.00	-2.97
8	MDI	Blood (P)	27.7	3.16	-0.21	-1.48
9	MDI	Blood (P)	15.6	3.16	2.14	-1.48
10	MDI	Blood (P)	26.5	3.16	-2.35	-2.97
11	MDI	AM Blood	29.6	3.16	2.35	-0.99
		AM Serum/Plasma	12.9	3.14	-0.64	-4.45
12	MDI	Blood (P)	17.2	3.14	-3.64	4.95
13	MDI	Blood (P)	41.5	3.15	-1.93	-1.48

DUID, driving under the influence of drugs; MDI, medicolegal death investigation; ND, Not detected; AM, antemortem; PM, postmortem; C, central; P, peripheral.

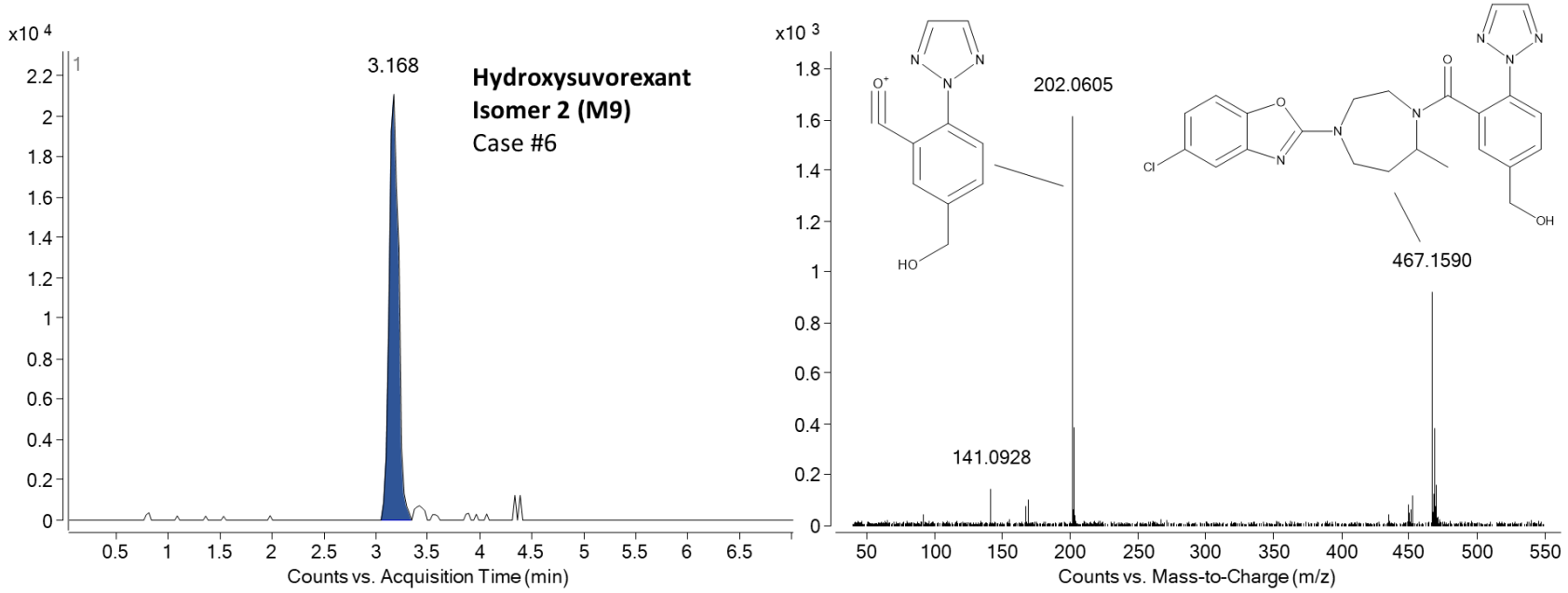


Figure 6.5. Extracted ion chromatogram (EIC) of a representative case sample (Case #6, 13.2 ng/mL suvorexant) containing the primary oxidative metabolite (M9).

Conclusions

The hydroxylated species M9 was confirmed as the primary phase I metabolite in unhydrolyzed blood samples. Although the metabolite is not believed to be pharmacologically active, drug/metabolite ratios can be of interpretive value in forensic toxicology, particularly medicolegal death investigation. However, quantitative analyses cannot be performed in the absence of a commercially available reference material.

Studies using rCYPs confirmed earlier reports regarding the involvement of CYP 3A4 and minor contributions of 2C19 (11). However, additional activity was identified for 2C9 and 2D6. The latter has implications in terms of genetic polymorphisms and the large interindividual differences observed in terms of enzyme activity. In-situ generated rCYP controls were used to confirm the presence of M9 in a series of thirteen suvorexant investigations. The metabolite was readily identified in 17 of the 18 specimens analyzed. This confirms earlier reports by Cui et al. that showed that suvorexant and the M9 metabolite accounted for 37% and 30% of the radiolabeled dose in plasma. As a first-in-class dual orexin receptor antagonist, suvorexant is a drug of forensic interest that should be considered during toxicological analyses.

Acknowledgments

We gratefully acknowledge Dr. Barry Logan (NMS Labs/Center for Forensic Science Research and Education) and Daniel Anderson, MS (Colorado Bureau of Investigation) for providing forensic specimens.

References

1. Cox, C.D., Breslin, M.J., Whitman, D.B., Schreier, J.D., McGaughey, G.B., Bogusky, M.J., et al. (2010) Discovery of the dual orexin receptor antagonist [(7R)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl]methanone (MK-4305) for the treatment of insomnia. *Journal of Medicinal Chemistry*. **53**, 5320-5332.
2. Hoyer, D. and Jacobson, L.H. (2013) Orexin in sleep, addiction and more: is the perfect insomnia drug at hand? *Neuropeptides*. **47**, 477-488.
3. Herring, W.J., Roth, T., Krystal, A.D. and Michelson, D. (2019) Orexin receptor antagonists for the treatment of insomnia and potential treatment of other neuropsychiatric indications. *Journal of Sleep Research*. **28**, e12782.
4. Citrome, L. (2014) Suvorexant for insomnia: a systematic review of the efficacy and safety profile for this newly approved hypnotic – what is the number needed to treat, number needed to harm and likelihood to be helped or harmed? *International Journal of Clinical Practice*. **68**, 1429-1441.
5. Coleman, P.J., Gotter, A.L., Herring, W.J., Winrow, C.J. and Renger, J.J. (2017) The discovery of suvorexant, the first orexin receptor drug for insomnia. *Annual Review of Pharmacology and Toxicology*. **57**, 509-533.
6. Herring, W.J., Snyder, E., Budd, K., Hutzelmann, J., Snavely, D., Liu, K., et al. (2012) Orexin receptor antagonism for treatment of insomnia: a randomized clinical trial of suvorexant. *Neurology*. **79**, 2265-2274.

7. U.S. Drug Enforcement Administration (2014) Schedules of Controlled Substances: Placement of Suvorexant into Schedule IV. Final Rule. *Federal Register*. **79**, 51243-51247.
8. Asai, Y., Sano, H., Miyazaki, M., Iwakura, M., Maeda, Y. and Hara, M. (2019) Suvorexant (Belsomra® Tablets 10, 15, and 20 mg): Japanese drug-use results survey. *Drugs in R&D*. **19**, 27-46.
9. Born, S., Gauvin, D.V., Mukherjee, S. and Briscoe, R. (2017) Preclinical assessment of the abuse potential of the orexin receptor antagonist, suvorexant. *Regulatory Toxicology and Pharmacology*. **86**, 181-192.
10. U.S. Food and Drug Administration (2013) Suvorexant Tablets Insomnia Indication, Peripheral & Central Nervous System Drugs Advisory Committee Meeting, NDA 204569. Available from: https://wayback.archiveit.org/7993/20170405225048/https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAdvisoryCommittee/UCM352970.pdf (accessed November 17, 2018).
11. Ciu, D., Cabalu, T., Lai Yee, K., Small, J., Li, X., Liu, B., et al. (2016) In vitro and in vivo characterisation of the metabolism and disposition of suvorexant in humans. *Xenobiotica*. **46**. 1-14.
12. Sun, H., Kennedy, W.P., Wilbraham, D., Lewis, N., Calder, N., Li, X., et al. (2013) Effects of suvorexant, an orexin receptor antagonist, on sleep parameters as measured by polysomnography in healthy men. *Sleep*. **36**, 259-267.

13. Rhyne, D.N. and Anderson, S.L. (2015) Suvorexant in insomnia: efficacy, safety and place in therapy. *Therapeutic Advances in Drug Safety*. **6**, 189-195.
14. Yang, L. (2014). Suvorexant: first global approval. *Drugs*. **74**, 1817-1822.
15. Merck & Co. (2014) Suvorexant prescribing information. Available from: http://www.merck.com/product/usa/pi_circulars/b/belsomra/belsomra_pi.pdf (accessed August 17, 2019).
16. Baselt, R.C. (2017) Suvorexant. In Disposition of toxic drugs and chemicals in man, 11th edition. Biomedical Publications, Seal Beach, CA, pp. 2005.
17. Keks, N.A., Hope, J. and Keogh, S. (2017) Suvorexant: scientifically interesting, utility uncertain. *Australasian Psychiatry*. **25**, 622-624.
18. Carson, M. and Kerrigan, S. (2017) Quantification of suvorexant in urine using gas chromatography/mass spectrometry. *Journal of Chromatography B*. **1040**, 289-294.
19. Breidinger, S.A., Simpson, R.C., Mangin, E. and Woolf, E.J. (2015) Determination of suvorexant in human plasma using 96-well liquid–liquid extraction and HPLC with tandem mass spectrometric detection. *Journal of Chromatography B*. **1002**, 254-259.
20. Iqbal, M., Ezzeldin, E., Khalil, N.Y., Al-Rashood, S.T.A. and Al-Rashood, K.A. (2017) Simple and highly sensitive UPLC-ESI-MS/MS assay for rapid determination of suvorexant in plasma. *Journal of Analytical Toxicology*. **41**, 114-120.
21. Iqbal, M., Khalil, N.Y., Ezzeldin, E. and Al-Rashood, K.A. (2018) Simultaneous detection and quantification of three novel prescription drugs of

abuse (suvorexant, lorcaserin and brivaracetam) in human plasma by UPLC–MS-MS. *Journal of Analytical Toxicology*. **43**, 203-211.

22. Iqbal, M., Ezzeldin, E., Khalil, N.Y., Alam, P. and Al-Rashood, K.A. (2019) UPLC-MS/MS determination of suvorexant in urine by a simplified dispersive liquid-liquid micro-extraction followed by ultrasound assisted back extraction from solidified floating organic droplets. *Journal of Pharmaceutical and Biomedical Analysis*. **164**, 1-8.
23. Sullinger, S., Bryand, K. and Kerrigan, S. (2017) Identification of suvorexant in urine using liquid chromatography-quadrupole/time-of-flight mass spectrometry (LC-Q/TOF-MS). *Journal of Analytical Toxicology*. **41**, 224-229.
24. Waters, B., Hara, K., Ikematsu, N., Takayama, M., Matsusue, A., Kashiwagi, M., et al. (2018) Tissue distribution of suvorexant in Three Forensic Autopsy Cases. *Journal of Analytical Toxicology*. **42**, 276-283.
25. Skillman, B., and Kerrigan, S. (2018) Quantification of suvorexant in blood using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry. *Journal of Chromatography B*. **1091**, 87-95.
26. Skillman, B. and Kerrigan, S. (2019) Identification of suvorexant in blood using LC-MS/MS: Important considerations for matrix effects and quantitative interferences in targeted assays. *Journal of Analytical Toxicology.* (In press). DOI 10.1093/jat/bkz083.
27. Li, F., Gonzalez, F.J. and Ma, X. (2012) LC–MS-based metabolomics in profiling of drug metabolism and bioactivation. *Acta Pharmaceutica Sinica B*. **2**, 118-125.

28. Lai, F.Y., Erratico, C., Kinyua, J., Mueller, J.F., Covaci, A. and van Nuijs, A.L.N. (2015) Liquid chromatography-quadrupole time-of-flight mass spectrometry for screening in vitro drug metabolites in humans: investigation on seven phenethylamine-based designer drugs. *Journal of Pharmaceutical and Biomedical Analysis*. **114** (Supplement C), 355-375.
29. Winborn, J., Haines, D., Kerrigan, S. (2018) *In vitro* metabolism of desomorphine. *Forensic Science International*. **289**, 140-149.

CHAPTER VII

Conclusion

Suvorexant is a novel insomnia medication with a unique mechanism of action. As a first-in-class dual orexin receptor antagonist, it is a drug of forensic interest. While few methods have been published regarding its detection in biological matrices, only one report to date described its distribution in postmortem fluids and tissues. As such, suvorexant's role in forensic investigations is relatively unknown and little information is available regarding its role in human performance toxicology. There are no reports detailing the analysis of suvorexant metabolites in casework, and some physicochemical properties of the drug are unstudied.

A method for the detection of suvorexant in whole blood was developed and validated using LC-Q/TOF-MS. Suvorexant was extracted from blood using a simple acidic/neutral LLE with *N*-butyl chloride. The LOD and LOQ were 0.5 ng/mL and a weighted (1/x) quadratic calibration model (R^2=0.995) over a concentration range of 2-200 ng/mL was used. Bias ranged from -5-2%, inter-assay precision (n=15) was 13%, 5%, and 8% for low, medium, and high concentrations, while intra-assay precision (n=5) was 10%, 4%, and 5%. Accuracy ranged from 95-102% over these concentrations. No carryover was observed, and mean matrix effects were 16% and 15% at low and high concentrations of suvorexant, respectively. Although no qualitative interferences from common drugs were observed, significant bias as a result of ion suppression was observed when sertraline was present at concentrations in excess of the internal standard, estazolam-D5. The interference was attributed to decreased ionization efficiency in the electrospray interface of the source

due to capacity limited ionization. These results further highlight the importance of stable isotope labeled internal standards, but these are not always commercially available.

The LC-Q/TOF-MS suvorexant method in blood was translated to, and cross-validated with LC-MS/MS. However, significant differences in matrix effects were observed for the two platforms, with LC-MS/MS matrix effects falling outside of validation guideline acceptability limits. The LC-MS/MS source parameters had been optimized using software, unlike the LC-Q/TOF-MS method which had been optimized manually prior to validation. Notably, this caused unacceptable values for matrix effects by LC-MS/MS, despite having an increased analytical response for suvorexant. Matrix effects were re-evaluated using various extraction solvents and commercial lipid clean-up devices which resulted in no observable differences. Applying the manually optimized LC-Q/TOF-MS parameters (i.e., de-tuning the source) in the LC-MS/MS assay eliminated the matrix effect, demonstrating how conditions at the electrospray interface can be manipulated to mitigate interferences. The LC-MS/MS method was subsequently validated and resulted in an LOD of 0.1 ng/mL and LOQ of 0.5 ng/mL in blood, respectively. The same calibration range was using (2-200 ng/mL) with a weighted (1/x) quadratic calibration model. Bias ranged from -2-2% and at low, medium, and high concentrations, intra-assay precision ranged from 4-7% and inter-assay precision ranged from 7-9%. Matrix effects were -8% and –9% at low and high concentrations of suvorexant using the new source conditions, and no carryover was observed. While no qualitative interferences were identified, there was a significant quantitative bias when sertraline and propoxyphene were present in concentrations exceeding that of the internal standard (estazolam-D5). The LC-MS/MS validation showed that both analytical platforms had comparable performance and were

sensitive for suvorexant at forensically relevant concentrations. Again, the utility of SIL-ISs was emphasized for the mitigation of potential quantitative interferences in targeted assays.

Given the limited literature describing the physicochemical properties of suvorexant and its unknown potential to undergo postmortem redistribution, partition coefficients of suvorexant were theoretically and experimentally determined to help characterize its lipophilicity. Theoretical partition coefficients were determined for suvorexant using ALogPS, ACD Labs/LogP, and KowWin predictive software which resulted in Log P values of 3.86, 3.62 ± 0.86, and 4.65, respectively. Experimental Log P values were determined using the shake-flask method using octanol/water and various aqueous buffer systems. The octanol/water partition coefficient was 3.45 ± 0.28 (n=3). Partitioning between octanol and various aqueous buffers with pH 2-9 and ionic strength from 10-200 mM resulted in Log P values of 3.23-4.04. Values were not significantly different (α= 0.01) using one-way ANOVA, consistent with its observed behavior as a neutral drug. Experimentally determined Log P values were in good agreement with those derived theoretically. Computational methods that made use of electrotopological estimations (ACD Labs/Log P, ALogPS) provided the closest values. Suvorexant was highly lipophilic, which could influence its behavior and disposition in the body before and after death.

Using the LC-Q/TOF-MS validated method, suvorexant was quantitated in specimens from a series of thirteen forensic investigations. The case specimens included antemortem and postmortem blood as well as serum/plasma and vitreous humor. Suvorexant was detected at concentrations ranging from 3-42 ng/mL, which are well within

the therapeutic range for suvorexant. C/P ratios were 2.0 and 2.2 for paired central and peripheral blood samples (n=2) which was in good agreement with the only published case report to date. Although inferences regarding PMR should be drawn from large populations, preliminary data on this new drug did not suggest significant postmortem redistribution.

An isotopically labeled internal standard was not commercially available at the time LC-Q/TOF-MS and LC-MS/MS methods were being validated. Using estazolam-D5 as the internal standard, significant quantitative biases were identified when certain other drugs were present in high concentrations. These drugs (sertraline and propoxyphene) coeluted with the internal standard and decreased ionization efficiency in the electrospray ionization source. Various strategies to mitigate this interference were explored which included decreasing the sample volume and the LC injection volume. It was determined that quantitative interferences could be improved when smaller sample and injection volumes were used. This indicating that as the number of ionizable species in the solvent droplet were decreased, there is less competition between the interfering species and the analyte of interest for desolvation in the source. Since the time of the original validation, a suvorexant SIL-IS (suvorexant-D6), became available and was evaluated in this interference study. It was determined that no interferences were observed using the original 52 common drugs, and quantitative interferences that resulted in unacceptable bias were completely mitigated. In addition, issues with dilution integrity experiments due to changes in the viscosity of the aqueous fraction were also ameliorated. This study highlighted the importance of evaluating method performance, particularly regarding matrix effects and drug-mediated interferences, qualitatively *and* quantitatively as well as using SIL-IS when available.

To date, only one published study has described the *in vivo* and *in vitro* metabolism of suvorexant. Despite the identification of oxidative metabolites in plasma, urine and principally feces, no commercially available reference standards are available, precluding metabolite quantification in forensic specimens. Recombinant CYPs (rCYP 3A4, 2C19, 2D6, 2C9, 2C8, 2C18, 2B6 and 1A2) were used to further investigate the metabolism of this new drug. Three phase I metabolites of suvorexant were identified using LC-Q/TOF-MS. Activity was demonstrated for a total of four rCYPs (3A4, 2C19, 2D6 and 2C9), all of which were confirmed using inhibitors. The involvement of 2C9, and in particular 2D6 (neither of which were identified previously), highlights the importance of additional studies.

The oxidative metabolite, M9, was identified using mass accuracy and MS/MS spectra. In-situ generated controls using rCYPs were used to confirm its presence in a series of thirteen forensic investigations. M9 was readily confirmed in seventeen of the eighteen specimens tested. Should a metabolite standard become available for this compound, parent-to-metabolite concentration ratios could be evaluated. These can be of use in medicolegal death investigations, particularly if a large bolus of drug is suspected, as may be the case following a suicide.

As suvorexant continues to be prescribed to patients diagnosed with insomnia, sensitive methods for its detection are needed. As a drug class, sedative hypnotics feature prominently in human performance toxicology (i.e., impaired driving, drug-facilitated sexual assault) and postmortem toxicology (i.e., medicolegal death investigations). Despite challenges associated with its detection, particularly using routine immunoassay-based or gas chromatographic-based screening, it should be considered. This research

provides forensic toxicology laboratories with sensitive and robust analytical methods for the quantitation of suvorexant using two different LC-MS platforms, and is the first to describe its analysis in blood using high resolution mass spectrometry. Moreover, physicochemical properties of suvorexant were explored which can help with interpretation of concentrations that are measured in various forensic specimens. This study adds to the limited literature regarding the analysis of suvorexant in authentic samples and is the first to describe the analysis of suvorexant metabolites in a forensic setting. The identification of new isoforms that might be responsible for metabolism is also a significant finding, particularly for CYP 2D6, which is known to be polymorphic. In addition to this new body of knowledge regarding suvorexant, important observations and limitations related to electrospray ionization were documented. Differences in assay performance between MS/MS and Q/TOF-MS platforms were identified, particularly as they related to matrix effects from endogenous species. Drug-mediated ion suppression was also explored and experimental approaches to mitigate these effects were investigated in order to improve the reliability and accuracy of quantitative measurements.

REFERENCES

ACD/LogP. Available from: https://www.acdlabs.com/products/percepta/predictors/logP/ (accessed August 17, 2019).

Andrés, A., Rosés, M., Ràfols, C., Bosch, E., Espinosa, S., Segarra, V., et al. (2015) Setup and validation of shake-flask procedures for the determination of partition coefficients (logD) from low drug amounts. *European Journal of Pharmaceutical Sciences.* **76,** 181-191.

Anzenbacher, P. and Anzenbacherova, E. (2001) Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences*. **58**, 737-747.

Asai, Y., Sano, H., Miyazaki, M., Iwakura, M., Maeda, Y. and Hara, M. (2019) Suvorexant (Belsomra® Tablets 10, 15, and 20 mg): Japanese drug-use results survey. *Drugs in R&D.* **19**, 27-46.

ASB/ANSI 098 Standard for Mass Spectral Data Acceptance in Forensic Toxicology-Draft (2019) Available from: https://www.nist.gov/sites/default/files/documents/2019/03/20/standard_for_mass_spec_spectral_data_acceptance_-_asb.pdf (accessed June 17, 2019).

Asha, S. and Vidyavathi, M. (2010) Role of human liver microsomes in in vitro metabolism of drugs-a review. *Applied Biochemistry and Biotechnology*. **160**, 1699-1722.

Atkin, T., Comai, S. and Gobbi, G. (2018) Drugs for insomnia beyond benzodiazepines: pharmacology, clinical applications, and discovery. *Pharmacological Reviews*. **70**, 197.

Baselt, R.C. (2017) Suvorexant. In Disposition of toxic drugs and chemicals in man, 11th edition. Biomedical Publications, Seal Beach, CA, pp. 2005.

Bennett, T., Bray, D. and Neville, M.W. (2014) Suvorexant, a dual orexin receptor antagonist for the management of insomnia. *P & T: A Peer-Reviewed Journal for Formulary Management*. **39**, 264-266.

Berg, T., and Strand, D. H. (2011) 13C labelled internal standards—A solution to minimize ion suppression effects in liquid chromatography–tandem mass spectrometry analyses of drugs in biological samples? *Journal of Chromatography A*. **1218**, 9366-9374.

Beuckmann, C.T., Ueno, T., Nakagawa, M., Suzuki, M. and Akasofu, S. (2019) Preclinical in vivo characterization of lemborexant (E2006), a novel dual orexin receptor antagonist for sleep/wake regulation. *Sleep*. **42**. (In press). https://doi.org/10.1093/sleep/zsz076

Born, S., Gauvin, D.V., Mukherjee, S. and Briscoe, R. (2017) Preclinical assessment of the abuse potential of the orexin receptor antagonist, suvorexant. *Regulatory Toxicology and Pharmacology*. **86**, 181-192.

Breidinger, S.A., Simpson, R.C., Mangin, E. and Woolf, E.J. (2015) Determination of suvorexant in human plasma using 96-well liquid–liquid extraction and HPLC with tandem mass spectrometric detection. *Journal of Chromatography B*. **1002**, 254-259.

Callander, G., Olorunda, M., Monna, D., Schuepbach, E., Langenegger, D., Betschart, C., et al. (2013) Kinetic properties of “dual” orexin receptor antagonists at OX1R and OX2R orexin receptors. *Frontiers in Neuroscience*. **7**, (230).

Carson, M. and Kerrigan, S. (2017) Quantification of suvorexant in urine using gas chromatography/mass spectrometry. *Journal of Chromatography B.* **1040**, 289-294.

Chambers, E., Wagrowski-Diehl, D. M., Lu, Z., and Mazzeo, J. R. (2007) Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography B.* **852**, 22-34.

Chen, Y., Zhou, Y., Li, J.-H., Sun, J.-Q. and Zhang, G.-S. (2015) Facile synthesis of suvorexant, an orexin receptor antagonist, via a chiral diazepane intermediate. *Chinese Chemical Letters*. **26**, 103-107.

Chou, J.T. and Jurs, P.C. (1979) Computer-assisted computation of partition coefficients from molecular structures using fragment constants. *Journal of Chemical Information and Computer Sciences*. **19**, 172-178.

Citrome, L. (2014) Suvorexant for insomnia: a systematic review of the efficacy and safety profile for this newly approved hypnotic – what is the number needed to treat, number needed to harm and likelihood to be helped or harmed? *International Journal of Clinical Practice*. **68**, 1429-1441.

Ciu, D., Cabalu, T., Lai Yee, K., Small, J., Li, X., Liu, B., et al. (2016) In vitro and in vivo characterisation of the metabolism and disposition of suvorexant in humans. *Xenobiotica*. **46**. 1-14.

Coleman, P.J., Gotter, A.L., Herring, W.J., Winrow, C.J. and Renger, J.J. (2017) The discovery of suvorexant, the first orexin receptor drug for insomnia. *Annual Review of Pharmacology and Toxicology*. **57**, 509-533.

Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, *Official Journal of the European Communities*, **L 221**, 8-36.

Cook, D.S., Braithwaite, R.A. and Hale, K.A. (2000) Estimating antemortem drug concentrations from postmortem blood samples: the influence of postmortem redistribution. *Journal of Clinical Pathology*. **53**, 282.

Cox, C.D., Breslin, M.J., Whitman, D.B., Schreier, J.D., McGaughey, G.B., Bogusky, M.J., et al. (2010) Discovery of the dual orexin receptor antagonist [(7R)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl]methanone (MK-4305) for the treatment of insomnia. *Journal of Medicinal Chemistry*. **53**, 5320-5332.

Donovan, S.F. and Pescatore, M.C. (2002) Method for measuring the logarithm of the octanol–water partition coefficient by using short octadecyl–poly(vinyl alcohol) high-performance liquid chromatography columns. *Journal of Chromatography A.* **952**, 47-61.

Drummer, O.H. (2004) Postmortem toxicology of drugs of abuse. *Forensic Science International*. **142**, 101-113.

Fang, N., Yu, S., Ronis, M.J. and Badger, T.M. (2015). Matrix effects break the LC behavior rule for analytes in LC-MS/MS analysis of biological samples. *Experimental biology and medicine*. **240**, 488-497.

Fujimoto, T., Kunitomo, J., Tomata, Y., Nishiyama, K., Nakashima, M., Hirozane, M., et al. (2011) Discovery of potent, selective, orally active benzoxazepine-based

Orexin-2 receptor antagonists. *Bioorganic & Medicinal Chemistry Letters*. **21**, 6414-6416.

Furey, A., Moriarty, M., Bane, V., Kinsella, B., and Lehane, M. (2013) Ion suppression; A critical review on causes, evaluation, prevention and applications. *Talanta*. **115**, 104-122.

Gentile, T.A., Simmons, S.J., Barker, D.J., Shaw, J.K., España, R.A. and Muschamp, J.W. (2018) Suvorexant, an orexin/hypocretin receptor antagonist, attenuates motivational and hedonic properties of cocaine. *Addiction Biology*. **23**, 247-255.

Ghosh, C., Shinde, C. P., and Chakraborty, B. S. (2012) Influence of ionization source design on matrix effects during LC–ESI-MS/MS analysis. *Journal of Chromatography B*. **893-894**, 193-200.

Gosetti, F., Mazzucco, E., Zampieri, D. and Gennaro, M.C. (2010) Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*. **1217**, 3929-3937.

Gotter, A.L., Winrow, C.J., Brunner, J., Garson, S.L., Fox, S.V., Binns, J., et al. (2013) The duration of sleep promoting efficacy by dual orexin receptor antagonists is dependent upon receptor occupancy threshold. *BMC Neuroscience*. **14**, 90.

Herring, W.J., Snyder, E., Budd, K., Hutzelmann, J., Snavely, D., Liu, K., et al. (2012) Orexin receptor antagonism for treatment of insomnia: a randomized clinical trial of suvorexant. *Neurology*. **79**, 2265-2274.

Herring, W.J., Connor, K.M., Ivgy-May, N., Snyder, E., Liu, K., Snavely, D.B., et al. (2016) Suvorexant in patients with insomnia: Results from two 3-month randomized controlled clinical trials. *Biological Psychiatry*. **79**, 136-148.

Herring, W.J., Roth, T., Krystal, A.D. and Michelson, D. (2019) Orexin receptor antagonists for the treatment of insomnia and potential treatment of other neuropsychiatric indications. *Journal of Sleep Research*. **28**, e12782.

Hoyer, D. and Jacobson, L.H. (2013) Orexin in sleep, addiction and more: is the perfect insomnia drug at hand? *Neuropeptides*. **47**, 477-488.

Iqbal, M., Ezzeldin, E., Khalil, N.Y., Al-Rashood, S.T.A. and Al-Rashood, K.A. (2017) Simple and highly sensitive UPLC-ESI-MS/MS assay for rapid determination of suvorexant in plasma. *Journal of Analytical Toxicology*. **41**, 114-120.

Iqbal, M., Khalil, N.Y., Ezzeldin, E. and Al-Rashood, K.A. (2018) Simultaneous detection and quantification of three novel prescription drugs of abuse (suvorexant, lorcaserin and brivaracetam) in human plasma by UPLC–MS-MS. *Journal of Analytical Toxicology*. **43**, 203-211.

Iqbal, M., Ezzeldin, E., Khalil, N.Y., Alam, P. and Al-Rashood, K.A. (2019) UPLC-MS/MS determination of suvorexant in urine by a simplified dispersive liquid-liquid micro-extraction followed by ultrasound assisted back extraction from solidified floating organic droplets. *Journal of Pharmaceutical and Biomedical Analysis*. **164**, 1-8.

Ismaiel, O.A., Halquist, M.S., Elmamly, M.Y., Shalaby, A. and Thomas Karnes, H. (2008) Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations. *Journal of Chromatography B*. **875**, 333-343.

Ismaiel, O.A., Zhang, T., Jenkins, R.G. and Karnes, H.T. (2010) Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry. *Journal of Chromatography B*. **878**, 3303-3316.

Iwatsubo, T., Hirota, N., Ooie, T., Suzuki, H., Shimada, N., Chiba, K., et al. (1997) Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. *Pharmacology and Therapeutics*. **73**, 147-171.

Janto, K., Prichard, J.R. and Pusalavidyasagar, S. (2018) An update on dual orexin receptor antagonists and their potential role in insomnia therapeutics. *Journal of clinical sleep medicine: JCSM: official publication of the American Academy of Sleep Medicine*. **14**, 1399-1408.

Janusson, E., Hesketh, A. V., Bamford, K. L., Hatlelid, K., Higgins, R., and McIndoe, J. S. (2015) Spatial effects on electrospray ionization response. *International Journal of Mass Spectrometry*. **388**, 1-8.

Jian, W., Edom, R.W., Xu, Y., Gallagher, J. and Weng, N. (2010) Potential bias and mitigations when using stable isotope labeled parent drug as internal standard for LC–MS/MS quantitation of metabolites. *Journal of Chromatography B*. **878**, 3267-3276.

Jones, G.R. and Pounder, D.J. (1987) Site Dependence of Drug Concentrations in Postmortem Blood—A Case Study*. *Journal of Analytical Toxicology*. **11**, 186-190.

Kah, M. and Brown, C.D. (2008) LogD: lipophilicity for ionisable compounds. *Chemosphere*. **72**, 1401-1408.

Kebarle, P., and Tang, L. (1993) From ions in solution to ions in the gas phase - the mechanism of electrospray mass spectrometry. *Analytical Chemistry*. **65**, 972A-986A.

Keks, N.A., Hope, J. and Keogh, S. (2017) Suvorexant: scientifically interesting, utility uncertain. *Australasian Psychiatry*. **25**, 622-624.

Kempińska, D., Chmiel, T., Kot-Wasik, A., Mróz, A., Mazerska, Z. and Namieśnik, J. (2019) State of the art and prospects of methods for determination of lipophilicity of chemical compounds. *TrAC Trends in Analytical Chemistry*. **113,** 54-73.

King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C. and Olah, T. (2000) Mechanistic investigation of ionization suppression in electrospray ionization. *Journal of the American Society for Mass Spectrometry*. **11**, 942-950.

Kripke, D. (2015) Is suvorexant a better choice than alternative hypnotics? *F1000Research*. **4,** 456.

Lai, F.Y., Erratico, C., Kinyua, J., Mueller, J.F., Covaci, A. and van Nuijs, A.L.N. (2015) Liquid chromatography-quadrupole time-of-flight mass spectrometry for screening in vitro drug metabolites in humans: investigation on seven phenethylamine-based designer drugs. *Journal of Pharmaceutical and Biomedical Analysis*. **114** (Supplement C), 355-375.

Larger, P.J., Breda, M., Fraier, D., Hughes, H. and James, C.A. (2005). Ion-suppression effects in liquid chromatography–tandem mass spectrometry due to a formulation agent, a case study in drug discovery bioanalysis. *Journal of Pharmaceutical and Biomedical Analysis*. **39**, 206-216.

Li, F., Gonzalez, F.J. and Ma, X. (2012) LC–MS-based metabolomics in profiling of drug metabolism and bioactivation. *Acta Pharmaceutica Sinica B.* **2**, 118-125.

Mangion, I.K., Sherry, B.D., Yin, J. and Fleitz, F.J. (2012) Enantioselective Synthesis of a Dual Orexin Receptor Antagonist. *Organic Letters*. **14**, 3458-3461.

Manier, S.K., Keller, A., Schäper, J. and Meyer, M.R. (2019) Untargeted metabolomics by high resolution mass spectrometry coupled to normal and reversed phase liquid chromatography as a tool to study the in vitro biotransformation of new psychoactive substances. *Scientific Reports*, **9**, 2741.

Matuszewski, B.K., Constanzer, M.L. and Chavez-Eng, C.M. (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*. **75**, 3019-3030.

McGuffey, J. E., Wei, B., Bernert, J. T., Morrow, J. C., Xia, B., Wang, L., et al. (2014) Validation of a LC-MS/MS Method for Quantifying Urinary Nicotine, Six Nicotine Metabolites and the Minor Tobacco Alkaloids—Anatabine and Anabasine—in Smokers' Urine. *PloS One*. **9**, e101816.

Medić-Šarić, M., Mornar, A., Badovinac-Črnjević, T., and Jasprica, I. (2004) Experimental and Calculation Procedures for Molecular Lipophilicity: A Comparative Study for 3,3'-(2-Methoxybenzylidene)bis(4-hydroxycoumarin). *Croatica Chemica Acta.* **77**, 367-370.

Medic-Saric, M., Mornar, A. and Jasprica, I. (2004) Lipophilicity study of salicylamide. *Acta Pharm.* **54**, 91-101.

Merck & Co. (2014) Suvorexant prescribing information. Available from: http://www.merck.com/product/usa/pi_circulars/b/belsomra/belsomra_pi.pdf (accessed August 17, 2019).

Michelson, D., Snyder, E., Paradis, E., Chengan-Liu, M., Snavely, D.B., Hutzelmann, J., et al. (2014) Safety and efficacy of suvorexant during 1-year treatment of insomnia with subsequent abrupt treatment discontinuation: a phase 3 randomised, double-blind, placebo-controlled trial. *The Lancet Neurology*. **13**, 461-471.

Mortier, K.A., Verstraete, A.G., Zhang, G.-F. and Lambert, W.E. (2004) Enhanced method performance due to a shorter chromatographic run-time in a liquid chromatography–tandem mass spectrometry assay for paclitaxel. *Journal of Chromatography A.* **1041**, 235-238.

National Institutes of Health (2005) NIH state-of-the-science conference statement on manifestations and management of chronic insomnia in adults. *NIH Consensus and State-of-the-Science Statements*. **22**, 1-30. https://consensus.nih.gov/2005/insomniastatement.pdf (accessed November 17, 2018).

Norman, J.L. and Anderson, S.L. (2016) Novel class of medications, orexin receptor antagonists, in the treatment of insomnia - critical appraisal of suvorexant. *Nature and science of sleep*. **8**, 239-247.

Pallicer, J.M., Calvet, C., Port, A., Rosés, M., Ràfols, C. and Bosch, E. (2012) Extension of the liquid chromatography/quantitative structure–property relationship method to assess the lipophilicity of neutral, acidic, basic and amphotheric drugs. *Journal of Chromatography A.* **1240**, 113-122.

Patel, K.V., Aspesi, A.V., Evoy, K.E. (2015) Suvorexant: a dual orexin receptor antagonist for the treatment of sleep onset and sleep maintenance insomnia. *Annals of Pharmacotherapy*. **49**, 477-483.

Pélissier-Alicot, A.-L., Gaulier, J.-M., Champsaur, P. and Marquet, P. (2003) Mechanisms Underlying Postmortem Redistribution of Drugs: A Review. *Journal of Analytical Toxicology*. **27**, 533-544.

Peters, F. T., and Remane, D. (2012) Aspects of matrix effects in applications of liquid chromatography–mass spectrometry to forensic and clinical toxicology—a review. *Analytical and Bioanalytical Chemistry*. **403**, 2155-2172.

Petrauskas, A. and Kolovanov, E. (2000) ACD/Log P method description. *Perspectives in Drug Discovery and Design.* **19**, 99-116.

Rhyne, D.N. and Anderson, S.L. (2015) Suvorexant in insomnia: efficacy, safety and place in therapy. *Therapeutic Advances in Drug Safety*. **6**, 189-195.

Rossmann, J., Gurke, R., Renner, L.D., Oertel, R. and Kirch, W. (2015) Evaluation of the matrix effect of different sample matrices for 33 pharmaceuticals by post-column infusion. *Journal of Chromatography B.* **1000**, 84-94.

Sakurai, T. (2013). Orexin deficiency and narcolepsy. *Current Opinion in Neurobiology*. **23**, 760-766.

Scientific Working Group for Forensic Toxicology (2013) Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. *Journal of Analytical Toxicology*. **37**, 452-474.

Shaw, P.N., Tan, S.K. and Hewavitharana, A. (2014) Strategies for the detection and elimination of matrix effects in quantitative LC–MS analysis. *LC GC North America.* **32**, 54-64. Available from: http://www.chromatographyonline.com/strategies-detection-and-elimination-matrix-effects-quantitative-lc-ms-analysis (accessed October 11, 2019).

Skillman, B., and Kerrigan, S. (2018) Quantification of suvorexant in blood using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry. *Journal of Chromatography B.* **1091**, 87-95.

Skillman, B. and Kerrigan, S. (2019) Identification of suvorexant in blood using LC-MS/MS: Important considerations for matrix effects and quantitative interferences in targeted assays. *Journal of Analytical Toxicology.* (In press). DOI 10.1093/jat/bkz083.

Skopp, G. (2010). Postmortem toxicology. *Forensic Science, Medicine, and Pathology.* **6**, 314-325.

Spessard, G.O. (1998) ACD Labs/LogP dB 3.5 and ChemSketch 3.5. *Journal of Chemical Information and Computer Sciences.* **38**, 1250-1253.

Strotman, N.A., Baxter, C.A., Brands, K.M.J., Cleator, E., Krska, S.W., Reamer, R.A., et al. (2011) Reaction development and mechanistic study of a ruthenium catalyzed intramolecular asymmetric reductive amination en route to the dual orexin inhibitor suvorexant (MK-4305). *Journal of the American Chemical Society*. **133**, 8362-8371.

Sullinger, S., Bryand, K. and Kerrigan, S. (2017) Identification of suvorexant in urine using liquid chromatography-quadrupole/time-of-flight mass spectrometry (LC-Q/TOF-MS). *Journal of Analytical Toxicology*. **41**, 224-229.

Sun, H., Kennedy, W.P., Wilbraham, D., Lewis, N., Calder, N., Li, X., et al. (2013) Effects of suvorexant, an orexin receptor antagonist, on sleep parameters as measured by polysomnography in healthy men. *Sleep*. **36**, 259-267.

Sun, H., Yee, K.L., Gill, S., Liu, W., Li, X., Panebianco, D., et al. (2015) Psychomotor effects, pharmacokinetics and safety of the orexin receptor antagonist suvorexant administered in combination with alcohol in healthy subjects. *Journal of Psychopharmacology*. **29**, 1159-1169.

Sun, H., Palcza, J., Rosenberg, R., Kryger, M., Siringhaus, T., Rowe, J., et al. (2015) Effects of suvorexant, an orexin receptor antagonist, on breathing during sleep in patients with chronic obstructive pulmonary disease. *Respiratory Medicine*. **109**, 416-426.

Sun, H., Palcza, J., Card, D., Gipson, A., Rosenberg, R., Kryger, M., et al. (2016). Effects of suvorexant, an orexin receptor antagonist, on respiration during sleep in patients with obstructive sleep apnea. *Journal of clinical sleep medicine: JCSM: official publication of the American Academy of Sleep Medicine*. **12**, 9-17.

Taka´cs-Nova´k, K. and Avdeel, A. (1996). Interlaboratory study of log P determination by shake-flask and potentiometric methods. *Journal of Pharmaceutical and Biomedical Analysis*. **14**, 1405-1413.

Taylor, P. J. (2005) Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry*. **38**, 328-334.

Tetko, I.V. and Tanchuk, V.Y. (2002) Application of Associative Neural Networks for Prediction of Lipophilicity in ALOGPS 2.1 Program. *Journal of Chemical Information and Computer Sciences.* **42**, 1136-1145.

Tetko, I.V. and Livingstone, D.J. (2007) 5.27 - Rule-Based Systems to Predict Lipophilicity. *Comprehensive Medicinal Chemistry II*. Taylor, J.B. and Triggle, D.J. Oxford, Elsevier**:** 649-668.

Trufelli, H., Palma, P., Famiglini, G., and Cappiello, A. (2011) An overview of matrix effects in liquid chromatography-mass spectrometry. *Mass Spectrometry Reviews*. **30**, 491-509.

Uemura, N., McCrea, J., Sun, H., Donikyan, M., Zammit, G., Liu, R., et al. (2015) Effects of the orexin receptor antagonist suvorexant on respiration during sleep in healthy subjects. *The Journal of Clinical Pharmacology*. **55**, 1093-1100.

U.S. Drug Enforcement Administration (2014) Schedules of Controlled Substances: Placement of Suvorexant into Schedule IV. Final Rule. *Federal Register*. **79**, 51243-51247.

U.S. Food and Drug Administration (2013) Suvorexant Tablets Insomnia Indication, Peripheral & Central Nervous System Drugs Advisory Committee Meeting, NDA 204569. Available from: https://wayback.archiveit.org/7993/20170405225048/https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCent

ralNervousSystemDrugsAdvisoryCommittee/UCM352970.pdf (accessed November 17, 2018).

Van Eeckhaut, A., Lanckmans, K., Sarre, S., Smolders, I. and Michotte, Y. (2009) Validation of bioanalytical LC–MS/MS assays: Evaluation of matrix effects. *Journal of Chromatography B*. **877**, 2198-2207.

Vermeeren, A., Sun, H., Vuurman, E.F.P.M., Jongen, S., Van Leeuwen, C.J., Van Oers, A.C.M., et al. (2015) On-the-road driving performance the morning after bedtime use of suvorexant 20 and 40 mg: A study in non-elderly healthy volunteers. *Sleep*. **38**, 1803-1813.

Vermeeren, A., Vets, E., Vuurman, E.F.P.M., Van Oers, A.C.M., Jongen, S., Laethem, T., et al. (2016) On-the-road driving performance the morning after bedtime use of suvorexant 15 and 30 mg in healthy elderly. *Psychopharmacology*. **233**, 3341-3351.

Villagrasa, M., Guillamón, M., Eljarrat, E. and Barceló, D. (2007) Matrix effect in liquid chromatography–electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material. *Journal of Chromatography A*. **1157**, 108-114.

Waters, B., Hara, K., Ikematsu, N., Takayama, M., Matsusue, A., Kashiwagi, M., et al. (2018) Tissue distribution of suvorexant in Three Forensic Autopsy Cases. *Journal of Analytical Toxicology*. **42**, 276-283.

Winborn, J., Haines, D., Kerrigan, S. (2018) *In vitro* metabolism of desomorphine. *Forensic Science International*. **289**, 140-149.

Winrow, C.J., Gotter, A.L., Cox, C.D., Doran, S.M., Tannenbaum, P.L., Breslin, M.J., et al. (2011) Promotion of sleep by suvorexant--A novel dual orexin receptor antagonist." *Journal of Neurogenetics*. **25**, 52-61.

Yang, L. (2014). Suvorexant: first global approval. *Drugs*. **74**, 1817-1822.

Zarrabian, S., Riahi, E., Karimi, S., Razavi, Y. and Haghparast, A. (2018) The potential role of the orexin reward system in future treatments for opioid drug abuse. *Brain Research*. (In press). https://doi.org/10.1016/j.brainres.2018.11.023

Zilg, B., Thelander, G., Giebe, B. and Druid, H. (2017) Postmortem blood sampling—Comparison of drug concentrations at different sample sites. *Forensic Science International*. **278**, 296-303.

APPENDIX

IRB Exemption Notices

Institutional Review Board
Office of Research and Sponsored Programs
1831 University Ave, Suite 303, Huntsville, TX 77341-2448
Phone: 936.294.4875
Fax: 936.294.3622
irb@shsu.edu
http://www.shsu.edu/dept/office-of-research-and-sponsored-programs/compliance/irb/

DATE:	March 26, 2018
TO:	Britni Skillman [Faculty Sponsor: Dr. Sarah Kerrigan]
FROM:	Sam Houston State University (SHSU) IRB
PROJECT TITLE:	*Suvorexant in Toxicological Investigations [T/D]*
PROTOCOL #:	2018-03-39109
SUBMISSION TYPE:	INITIAL REVIEW
ACTION:	DETERMINATION OF EXEMPT STATUS
DECISION DATE:	March 26, 2018
REVIEW CATEGORY:	Category 4—research involving existing, publicly available data usually has little, if any, associated risk, particularly if subject identifiers are removed from the data or specimens.

Thank you for your submission of Initial Review materials for this project. The Sam Houston State University (SHSU) IRB has determined this project is EXEMPT FROM IRB REVIEW according to federal regulations.

We will retain a copy of this correspondence within our records.

*** What should investigators do when considering changes to an exempt study that could make it nonexempt?**

It is the PI's responsibility to consult with the IRB whenever questions arise about whether planned changes to an exempt study might make that study nonexempt human subjects research. In this case, please make available sufficient information to the IRB so it can make a correct determination.

If you have any questions, please contact the IRB Office at 936-294-4875 or irb@shsu.edu. Please include your project title and protocol number in all correspondence with this committee.

Sincerely,

Donna Desforges
IRB Chair, PHSC

This letter has been electronically signed in accordance with all applicable regulations, and a copy is retained within Sam Houston State University IRB's records

VITA

Britni Skillman

Relevant Professional Experience

Sam Houston State University

August 2015- Present

- Graduate Assistant to Dr. Sarah Kerrigan and Dr. Sparks Veasey
- Aided and mentored in various toxicology projects, instrument maintenance and troubleshooting, laboratory housekeeping, inventory, and administrative duties.

U.S. Drug Enforcement Administration (DEA) South Central Laboratory

May 2016-August 2016

- Student Intern (Forensic Chemistry)
- Attended chemist training and lecture series including, but not limited to, Annual Clandestine Lab Recertification, Drug Trends for Emergency Care Personnel, Drugs Trends and Drug Manufacturing/Processing, Drugs Trends and Synthetic Pharmacology, Fire Drill & Extinguisher training, and Analytical Schemes of Synthetics
- Worked closely with the quality assurance personnel to learn responsibilities relating to the maintenance and verification of instruments and assisted in Level 2 method validations for LC instrumentation
- Assisted chemists with bulk cases, including stripping bricks of cocaine and other bulk exhibits of their original packaging, needle probing exhibits for screening by GC/MS, performing color tests, and conducting FTIR analyses of unknown powders
- Worked in the evidence vault performing a bi-monthly evidence review to prepare for destruction events of closed cases or those which need not be analyzed
- Gained experience with LC-Q/TOF-MS:
    - Performed quadrupole checks, TOF mass calibrations, system tunes, instrument cleaning and maintenance, and data input for compound database building
    - Created a preliminary method for the analysis of psilocin and psilocybin

- Helped develop a screening method for 10 synthetic compounds (4-APB, 5-APB, 6-APB, 7-APB, 2-MAPB, 5-MAPB, 5-APDB, 6-APDB, 5-fluoro-MDMB PINACA, and MMB-CHMICA) with the ability to resolve the structural isomers

Education

Sam Houston State University

August 2015- Present

- Pending Doctor of Philosophy in Forensic Science
- Focus: Forensic toxicology
- Expected Graduation: December 2019

Sam Houston State University

- B.S. in Forensic Chemistry and Criminal Justice
- Minor: Biology
- Graduated Cum Laude and with honors from Elliot T. Bowers Honors College

Relevant Educational Experience

Sam Houston State University

- Graduate: Forensic Toxicology, Forensic Instrumental Analysis, Advanced Instrumental Analysis, Drug and Toxin Biochemistry, Controlled Substance Analysis, Neuropsychopharmacology, Advanced Forensic Chemistry, Forensic Statistics and Interpretation
- Undergraduate: Forensic Chemistry, Instrumental Analytical Chemistry, Quantitative Analysis, Advanced Inorganic Chemistry, Introductory Biochemistry, Physical Chemistry I, Chemical Literature Seminar, Discoveries in Chemistry & Textiles, Organic Chemistry I & II, General Chemistry I & II

Skills and Qualification

Extraction and Screening Techniques

- Proficient with liquid-liquid extraction (LLE), solid phase extraction (SPE), protein precipitation, and commercial lipid removal devices
- Familiar with enzyme-linked immunosorbent assays (ELISA), color tests, and FTIR screening

Instrumentation

- Proficient with Liquid Chromatography-Quadrupole/Time-of-Flight-Mass Spectrometry (LC-Q/TOF-MS), Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), and Gas Chromatography-Mass Spectrometry (GC-MS).
- Familiar with Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR), Ion Mobility Spectroscopy (IMS), High Performance Liquid Chromatography (HPLC), Video Spectral Comparator (VSC), and microscopy

Software

- Agilent MassHunter Acquisition, Qualitative Analysis, and Quantitative Analysis
- ChemStation
- ACD ChemDraw/ChemSketch
- ACD Labs/LogP
- LogKowWin
- ALogPS 2.1
- R Statistical Software

Peer-Reviewed Publications

Skillman, B. and Kerrigan, S. (2018) Quantification of suvorexant in blood using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry. *Journal of Chromatography B*. 1091, 87-95.

Skillman, B. and Kerrigan, S. (2019) Identification of suvorexant in blood using LC-MS/MS: Important considerations for matrix effects and quantitative interferences in targeted assays. *Journal of Analytical Toxicology*. (In press). DOI 10.1093/jat/bkz083.

Skillman, B. and Kerrigan, S. (2019) Molecular lipophilicity of suvorexant using experimental and theoretical estimates: Implications for forensic toxicology. *Journal of Forensic Sciences*. (In review).

Skillman, B. and Kerrigan, S. (2019) Drug-Mediated Ion Suppression of Suvorexant and Mitigation of Interferences Using Liquid Chromatography-Quadrupole/Time of Flight Mass Spectrometry (LC-Q/TOF-MS) and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). *Journal of Chromatography B*. (In review).

Skillman, B. and Kerrigan, S. (2019) CYP450-mediated metabolism of suvorexant and investigation of metabolites in authentic case specimens. *Forensic Science International*. (In review).

Peer-Reviewed Presentations and Posters

Skillman, B. and Kerrigan, S. Comparison of Physicochemical Properties of Suvorexant with Quantitative Results using Authentic Samples. POSTER PRESENTATION. Proceedings of the Society of Forensic Toxicologists (SOFT) annual meeting in San Antonio, TX (2019).

Skillman, B. and Kerrigan, S. Side-by-Side Comparison of LC-Q/TOF-MS and LC-MS/MS Validation Parameters for the Detection of Suvorexant in Blood. ORAL PRESENTATION. Proceedings of the Southwestern Association of Toxicologists (SAT) annual meeting in Houston, TX (2019).

Skillman, B. and Kerrigan, S. Side-by-Side Comparison of LC-Q/TOF-MS and LC-MS/MS Validation Parameters for the Detection of Suvorexant in Blood. ORAL

PRESENTATION. Proceedings of the Society of Forensic Toxicologists (SOFT) annual meeting in Minneapolis, MN (2018).

Skillman, B., Bryand, K., and Kerrigan, S. Quantification of Suvorexant in Blood Using LC-Q/TOF-MS. POSTER PRESENTATION. Proceedings of the SOFT/TIAFT joint meeting in Boca Raton, FL (2017).

Other Reports

Bravenec, V. and **Skillman. B.** (July 2016) Quantitation of Cocaine-Quantitative Method Validation Final Report- Level 2. U.S. Drug Enforcement Administration- South Central Laboratory, Office of Forensic Sciences. Method # DEA 201. Report # QMV-201-2-361408.

Bravenec, V. and **Skillman. B.** (July 2016) Quantitation of Heroin- Quantitative Method Validation Final Report- Level 2. U.S. Drug Enforcement Administration- South Central Laboratory, Office of Forensic Sciences. Method # DEA 202. Report # QMV-202-2-361408.

Professional Affiliations and Memberships

- Society of Forensic Toxicologists (SOFT)- Student Member (2017-present).
- American Academy of Forensic Sciences (AAFS)- Student Member (2015-present).
- American Chemical Society (ACS)- Student Member (2015-present).
- Society of Forensic Science at SHSU- Vice president (Fall 2017-Fall 2019), Media Coordinator (Fall 2015-Fall 2017), Undergraduate Student Member (2014-2015).

Awards

- SOFT 2019 Leo Dal Cortivo Award- Best Poster Presentation
- Sparks Veasey, III, MD, JD Memorial Prize (Spring 2019)
- LTC Michael A. Lytle ’77 Academic Prize in Forensic Science (Spring 2017)
- O.B. Ellis & J.P. Gibbs Memorial Scholarship (Spring 2016)

Continuing Education

- OSHA Certification in Blood Borne Pathogens and Laboratory Standard
- RTI International Forensic Science Education
    - Agilent 2019 Online Symposium: Sample Preparation, Instrument Method Development, Forensic Toxicology Method Validation, Seized Drugs
- Forensic Technology Center of Excellence
    - NIJ and RTI: Regional Fentanyl Trends, Safety, and Field Testing Technology
- DEA South Central Laboratory
    - Drugs Trends for Emergency Care Personnel
    - Drug Trends and Drug Manufacturing/Processing
    - Drug Trends and Synthetic Pharmacology
    - Analytical Schemes of Synthetics
    - Records and Information Management Course I
    - Mandatory Respiratory Protection
    - DEA Environmental Management System Awareness Training