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Postmortem Distribution of Zolpidem in Aviation Accident Victims

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Kristi S. Thompson

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Postmortem Distribution of Zolpidem in Aviation Accident Victims

By: Kristi S. Thompson

A THESIS

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By _____

Dr. David von Minden Committee Chair

_____

Dr. Russell J. Lewis Committee Member

Robert O. Bost, Ph.D., DABFT

Dr. Robert O. Bost Committee Member

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Abstract

The purpose of the study was to confirm there is a consistent postmortem distribution of zolpidem in postmortem blood and body tissues, and/or fluids making it possible to estimate zolpidem blood concentrations from fluid and tissue concentrations. The samples for the study were zolpidem-positive fatalities located in the CAMI ToxFlo database at the FAA's Bioaeronautical Sciences Research Laboratory. A UPLC-MS method was developed and validated. The LDR, LOD, LOQ, accuracy, precision, and matrix effect were evaluated. The specimens were analyzed for the distribution of zolpidem. The results of this study confirmed there is a consistent postmortem distribution of zolpidem found in the spleen, kidney, and lung; making it possible to roughly estimate, zolpidem blood concentrations from these tissues.

TABLE OF CONTENTS

List of Tables

List of Figures

Chapter I. Introduction

Aviation accidents are violent events, often involving fatalities. When an aviation accident occurs, an investigation is necessary to determine the cause of the accident. A part of this investigation includes a toxicological evaluation of the pilot and other crew members to determine if drugs or alcohol were a contributing factor in the accident. Following an aviation accident, specimens are collected and forwarded to the Bioaeronautical Sciences Research Laboratory at the Federal Aviation Administration's (FAA) Civil Aerospace Medical Institute (CAMI, Oklahoma City, OK) for toxicological analysis.

Due to the traumatic nature of aviation accidents, the bodies of the victims may be badly dismembered, fragmented and/or burned. While therapeutic concentrations of drugs are generally reported in blood or plasma, there may be no blood available for analysis; thus, only tissues available for toxicological use and interpretation.

Statement of the Problem

In the cases received by the FAA's Bioaeronautical Sciences Research Laboratory only approximately 70% include blood specimens; therefore, the laboratory must rely on tissue samples in 30% of the cases for interpretive purposes (Lewis, Johnson, Southern, & Canfield, 2003). It may be possible to estimate blood concentrations of a drug from tissue concentrations by understanding the postmortem distribution of the drug. Postmortem distribution is especially important for impairing drugs, since such drugs are capable of aviation accident causation. The sleep aid, zolpidem, is such a drug.

Zolpidem is one of the most widely prescribed drugs in North America. In fact, zolpidem is the number one sleep aid prescribed in North America. With such prevalence, the likelihood of zolpidem being identified in a pilot involved in an aviation accident is great. Therefore, it was necessary to determine whether or not zolpidem was a drug that had the ability to negatively impact a pilot's ability to safely fly an aircraft. Since the FAA laboratory relies on postmortem samples to evaluate ante mortem "conditions", it was important to determine what existing research, if any; pertaining to the postmortem aspects of zolpidem had been completed. Finally, postmortem analysis is very difficult due to the use of tissues and the putrefied state of many specimens. It was necessary to determine which methodology would be best suited for providing valid, reliable, and accurate analytical results in such specimens.

The first two areas are related in making a determination as to whether or not additional research in the postmortem distribution of zolpidem was relevant and needed. The third area focuses on how this research would be analytically performed. All three areas are related to the postmortem distribution of zolpidem.

Background and Need

The first topic to be considered was whether or not it was necessary to study the impairing drug, zolpidem. The pilot's ability to safely command an aircraft is effected by the medication(s) he/she takes. The issue of concern was aviation safety. The second topic to be considered was whether previous postmortem zolpidem studies had been performed, to avoid a duplication of efforts. The third topic was the methodology to be used for the analysis of

zolpidem in postmortem specimens. All three of these areas had to be researched before a feasible plan for the study could be developed.

Zolpidem is a nonbenzodiazepine sedative hypnotic drug used to treat insomnia by slowing the activity in the brain. It is to be used as a short-term treatment for insomnia and has been used in the United States since 1993 (Rohrig & Moore, 2005). Zolpidem is an imidazopyridine derivative with the chemical name N,N-6-trimethyl-2-(4-methylphenyl)imidazo[1,2-α] pyridine-3-acetamide and molecular weight of 307.4 (see Figure 1). It is a Schedule IV controlled substance, also known as Ambien. Ambien is available in 5 mg white oval tablets and 10 mg pink oval tablets. Zolpidem is a crystalline powder, white to off-white in color. The recommended dosage is 10 mg just prior to bedtime. The dosage for the elderly is generally 5 mg (Couper & Logan, 2010).

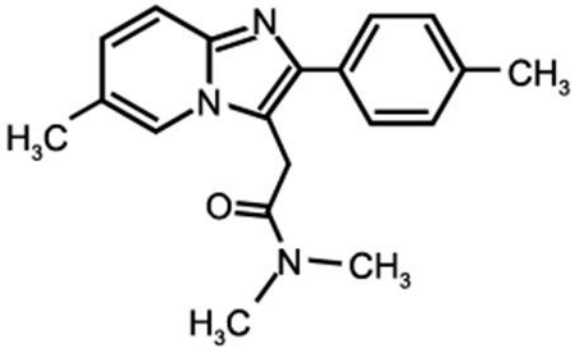

Figure 1. Chemical structure of zolpidem, $C_{19}H_{21}N_3O$.

Zolpidem is readily absorbed from the gastrointestinal tract. Zolpidem is also quickly eliminated, with a half-life of approximately 2.5 hr (Baselt, 2002). Research has shown that the

half-life is less in children and greater in the elderly (Couper & Logan, 2010). Peak concentrations in plasma are noted at approximately 1.6 hr (Baselt, 2002). Concentrations are less when taken with food. Blood concentrations are dose dependent, averaging 0.06 mg/L at 1.6 hr for a 5 mg dose and are doubled for the 10 mg dose for 0.12 mg/L at 1.6 hr (Couper & Logan, 2010). The therapeutic range for zolpidem in blood is 0.08-0.15 mg/L (Uges, 2004). Its toxic range begins at 0.5 mg/L and the lethal range begins at 2-4 mg/L (Uges, 2004).

There are many effects from zolpidem. These include psychological effects ranging from drowsiness and dizziness to amnesia and memory impairment. Physiological effects range from nausea to slow and slurred speech, diminished reflexes and a lack of coordination. Other side effects include, but are not limited to, nightmares, hallucinations, leg cramps, double vision, and dry mouth. An oral dose of 10-20 mg can have effects lasting up to 4-5 hr, and may last as long as 8-16 hr if concurrent use of other central nervous system (CNS) depressant drugs is involved (Couper & Logan, 2010).

There are numerous case reports of accidental consumption of zolpidem. Two cases reported, both being physicians involved a 50 year old male and a 43 year old male. In both cases, the subjects accidentally ingested a 10 mg Ambien pill during the day, under similar circumstances. They were both away from home, had put all of their pills in the same bottle and took the wrong pill. Both experienced 3 to 5 hr of symptoms such as amnesia, confusion, and ataxia. Their spouses took care of them until the symptoms passed (Poceta, 2011). A 28 year old female took her antidepressant at night and Ambien in the morning for several days and was seen with the same symptoms of confusion and ataxia. She switched the Ambien with the 100 mg Zoloft which was similar in shape and color (Poceta, 2011).

In a study with 29 subjects arrested for impaired driving, five of the cases listed zolpidem as the only drug. The signs of impairment in the five cases were slow reflexes, slow and slurred speech, disorientation, lack of balance and coordination, and "blacking out" (Logan & Couper, 2001). As Logan and Couper (2001) indicated, blood concentrations consistent with therapeutic doses of zolpidem can affect driving, and concentrations above the normal therapeutic range would increase driving impairment. This is due to zolpidem's activity as a sleep inducer (Logan & Couper, 2001).

A thorough review of the literature supported the impairing effects of zolpidem, which validates the original assertion that zolpidem could affect a pilot's ability to safely fly an aircraft. The side effects of zolpidem can substantially affect a pilot's performance and could be a contributory factor in an aviation accident (Johnson, Lewis, & Angier, 2007).

A survey of the literature was completed for studies relating to postmortem distribution of zolpidem. Only one distribution study was identified; however, of the five cases reported, four had toxic levels and one had a lethal level of zolpidem (Takayas, Ishida, Kimura, Kawaguchi, & Kondo, 2008). The proposed research project will evaluate cases in which zolpidem concentrations will likely be in the therapeutic range, as opposed to the toxic/lethal which exhibit abnormal pharmacokinetics and distribution. Furthermore, a complete postmortem distribution will be evaluated, including the analysis of blood, urine, vitreous humor, liver, lung, kidney, spleen, muscle, and heart. No other studies of a similar nature were identified. Performing this study was necessary to fill the gap in knowledge of the postmortem distribution of zolpidem.

Zolpidem can be analyzed by several different analytical techniques, including immunoassay, gas chromatography-mass spectrometry (GC-MS), high performance liquid

chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) (Nielsen & Johansen, 2012). GC-MS, LC-MS-ion trap and UPLC-MS were evaluated with respect to zolpidem, both in the literature and laboratory. UPLC-MS was selected based on its sensitivity and rapid analysis time compared to other tested analytical techniques. This decision will be discussed in-depth in Chapter 5.

The three topics of concern confirmed the necessity of the proposed research and the availability of methodology for accurate determination of zolpidem in postmortem specimens. The first area showed zolpidem had the potential to impair a pilot's ability to fly an aircraft. The second area demonstrated the need for additional studies in the area of postmortem distribution, as there was no study available for zolpidem at therapeutic concentrations that included the broad specimen types proposed here. The third area determined UPLC-MS analysis would be the best instrument to perform this study. In the next section, the purpose for performing this study will be discussed.

Purpose of the Study

The purpose of this study was to determine the correlation, if any, in the postmortem distribution of zolpidem between blood and a variety of body fluids and tissues. This research was proposed to provide the forensic community a set of tools to aid in estimating the concentration of zolpidem in postmortem blood from tissues, when no blood is available. Without this study, in the absence of blood, it would not be possible to make any determination

as to whether or not the drug, zolpidem, if found in the tissues made any contribution for the cause of the accident.

This study was performed by identifying positive zolpidem cases with body tissues and fluids, including blood. A method was developed and validated to analyze the postmortem samples. The limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision, and the matrix effect were evaluated and will be discussed in detail in Chapter 5. Finally, the specimens were analyzed for the distribution of the drug. The hypothesis of this study was that there is a blood-tissue correlation that can be used to estimate zolpidem blood concentrations from tissue concentrations in pilots and/or crew from fatal aviation accidents.

Research Questions

There were two research questions to be addressed with this study. Was there a consistent distribution of zolpidem in postmortem blood and unique body tissues, and/or other body fluids? If so, would it then be possible to estimate zolpidem blood concentrations from the zolpidem fluid and tissue concentrations?

Significance to the Field

There were no short or long term benefits for the participants in the study as they were all deceased. The benefit derived from this study was the possibility of being able to determine whether zolpidem could be a factor in an aviation accident, when there was no blood available from the deceased victims. From the results of this particular study, it is possible, with extreme

caution, to use some tissue concentrations to estimate blood concentrations; this may help determine if zolpidem was in the therapeutic, toxic, or lethal range.

Definitions

Atmospheric pressure chemical ionization (APCI): APCI is a "soft" ionization technique used in LC-MS where mainly protonated or deprotonated molecular ions are formed. The sample is vaporized in a heated nebulizer before emerging into plasma consisting of solvent ions formed within the atmospheric source by a corona discharge. Proton transfer or abstraction then takes place between the solvent ions and the sample. It provides molecular mass information for compounds that have some volatility and are of medium polarity. APCI is used to analyze heat-stable, small molecules and only allows for single charging due to the ionization mechanism. It is a robust technique and normally not affected by changes in buffer type.

Ataxia: lack of coordination of voluntary muscle movements.

Binary solvent manager (BSM): high pressure pumps that move up to two solvents simultaneously through the LC system.

Electrospray Ionization (ESI): ESI is a "soft" ionization technique used in LC/MS which transfers ions from liquid solutions into the gas phase. The sample solution enters the ESI needle where it is contacted with high voltage, the needle then sprays the sample solution into a fine mist of electrically charged droplets. The droplets then divide into smaller droplets repeatedly. As solvent evaporates, the charge density increases and the droplet size decreases. From the highly charged, very small droplets, sample ions are ejected into the gas phase by electrostatic repulsion. The sample ions then pass through an ion transfer capillary and enter the MS detector

where they are analyzed. ESI is able to analyze many samples which were previously not suitable for mass analysis such as, high molecular mass and heat-labile compounds. ESI is also used to analyze polar and semi-polar compounds.

Liquid Chromatography (LC): Liquid chromatography separates the components of a sample based on differences in their affinity or attraction for the stationary or mobile phase. The separated components are then detected by UV, fluorescence, or electrical conductivity based on their properties. The detectors identify substances based on retention time and quantitate them based on peak intensity and area.

Mass spectrometry (MS): Mass spectrometry has a highly sensitive detection technique that ionizes sample components then separates the ions in vacuum based on their mass-to-charge ratios and measures the intensity of each ion. MS provides mass spectra that can indicate the concentration level of ions that have a given mass.

Matrix effect: the combined effect of all components of the sample other than the analyte on the measurement of the quantity of an analyte.

Schedule IV controlled substance: drugs/chemicals with a low potential for abuse and low risk of dependence.

Therapeutic level: when a drug in the bloodstream is in the range in which the drug is effective without causing any serious problems to the patient.

Toxic level: concentrations above the acceptable level when side effects and toxic effects may appear.

Toxicological analysis: detecting, identifying, and quantitating substances relative to toxicology.

Ultra performance liquid chromatography (UPLC): a technology advance in high performance liquid chromatography instrumentation and columns, resulting in increased resolution, speed, and sensitivity.

Xenobiotics: an exogenous chemical or substance found in an organism which is not usually found or expected to be there.

Limitations

The small sample size was a limitation of this study, which would lessen the internal validity of the project. There were numerous zolpidem-positive cases identified in the CAMI database; however, only 10 cases had a majority of the desired tissues and fluids needed for the research study. Another limitation in this study was the lack of autopsy information. The blood collection site(s) and postmortem interval for these cases are unknown. However, in most of the cases received at the Bioaeronautical Sciences Research Laboratory where the collection site is reported, the blood typically is noted as having been collected from the chest cavity. This project is not high in external validity because the samples are coming from participants who are deceased, with the probable cause of death being from blunt force trauma, i.e., aviation accident.

Ethical Considerations

An application for institutional review board (IRB) research approval, entitled Postmortem Distribution of Zolpidem in Aviation Accident Victims, was submitted to the University Of Central Oklahoma (UCO) IRB. On April 16, 2010, a letter was received from Jill

A. Devenport, Ph.D., Chair of the IRB at UCO, indicating that because the samples being used for this research were obtained from deceased individuals, the research was exempt from IRB review. There were other legal and ethical issues outside the scope of the IRB which were to be reviewed by UCO's General Counsel. UCO's General Counsel prepared a Waiver of Liability and Hold Harmless Agreement that was signed by Kristi S. Thompson, Participant, on January 21, 2011; David L. von Minden, Thesis Instructor; and Dr. Dwight E. Adams, Director, Forensic Science Institute, on January 24, 2011. An annual status follow-up on IRB #10075 was received in March 2012 and another in March 2013 from Pam Lumen, Coordinator, at the Office of Research Compliance at UCO. Kristi S. Thompson, Participant, notified UCO, following each request, indicating that the research is still ongoing and that no changes have been made to the research plan which would change the IRB exemption status.

Chapter II. Literature Review

A pilot's ability to safely fly a plane is a key part of aviation safety. One way to assure aviation safety is providing the pilot with up to date information about prescription medications and their effects. Determining the postmortem distribution of a drug increases the drug knowledge base and provides information for FAA flight guidelines. A study of positive drug findings from pilot fatalities over a five year period was completed by the FAA. This study was a continuation of three prior five-year studies. Zolpidem was a new drug appearing in the study from 1999-2003. Two positive findings for zolpidem were identified in that five year period accounting for 0.1% of the total number of drug positives. In the next five-year study, 2004-2008, the number increased to seven positive zolpidem cases accounting for 0.5% of the total number of drug positives (Canfield, Dubowski, Chaturvedi, & Whinnery, 2011). Zolpidem being a relatively new drug on the market with rising positive findings in pilots made it a drug to consider studying its postmortem distribution.

This literature review will address three areas related to the postmortem distribution of zolpidem. Each of the three sections will consist of a review of three journal articles. The first section will address research related to the effects of zolpidem. The second will address research about the postmortem distribution of zolpidem used here to design a relevant study. The third section will address the analytical methodology to be used in performing the proposed study. The articles support the need for the proposed postmortem distribution study of zolpidem.

Is Zolpidem a Drug Which Can Affect a Pilot's Ability to Fly a Plane?

Zolpidem has been associated with sleep-walking, amnesia, sleep-eating, and sleep-driving when taken at night. It has been known to impair cognition, memory, and motor performance when ingested during the daytime. There has been a high frequency of zolpidem detected in the blood of people apprehended for driving under the influence (DUI) (Poceta, 2011).

Describing daytime automatisms (amnesia) and sleep-related parasomnias (sleep-driving), both being zolpidem-associated complex behaviors, was the purpose of this study (Poceta, 2011). Poceta's (2011) study was based on a case review composed of eight clinical and six legal cases. The participants had either a history of ingesting zolpidem or a positive zolpidem result from a drug analysis. The study took place in La Jolla, California, at the Scripps Clinic. The clinical participants were patients seen for symptoms of confusion, amnesia, or sleep-walking. The legal participants were defendants from legal cases being prosecuted for DUI. Dr. Poceta was compensated as an expert witness evaluating the records for the defense on each case. The participants were both male and female, ranging in age from 28-65 for the clinical patients and 33-54 for the legal defendants.

The data was extracted from the clinical case reports and placed into categories by age, gender, Body Mass Index (BMI), time of zolpidem ingestion, behavior, dose taken, other medications taken, and any known sleep disorders. Data from the legal cases were placed into categories by age, gender, behavior, dose, concentration (ng/mL) of zolpidem and other medications found by toxicological analysis, and comments as to the case results.

The results of the eight clinical case studies indicated four of the cases were accidental ingestion of zolpidem during the day, all with amnesia type behavior. The patient in the first of the accidental ingestion cases was a man on vacation; he had put all of his pills in the same bottle and took two 10 mg zolpidem tablets instead of two prednisone tablets on the date of the symptoms. Two of the cases were physicians who were also on vacation and each put their pills in a bottle, taking the wrong pill in the morning. The fourth case was a woman who accidentally took her antidepressant at night and her sleeping pill in the morning. No sleep disorder was indicated in any of the four accidental ingestion cases. Two of the four cases included positive results for other intervening medications. In another case, the subject took zolpidem during the day for headaches resulting in both amnesia and inebriated behavior. Two other medications were being taken at the same time. There was no sleep disorder associated with the case. The remaining three clinical cases all indicated zolpidem was ingested at bedtime. All three subjects from these cases had known sleep disorders such as snoring, periodic limb movement syndrome (PLMS), or obstructive sleep apnea (OSA). Two of the case histories noted the subjects were taking other medications. Their resulting behavior varied from sleep-driving, sleep-walking, and sleep-eating to flooding the residence while taking a bath.

The results of the six legal cases all involved sleep driving. All but one of the cases involved other medications or alcohol. One case involved a dosage over the normal range. Another involved no known dosage; however, all of the cases had zolpidem results over the therapeutic level.

It was reported that all of the patients and defendants showed signs of abnormal behavior with poor motor control, confusion and amnesia for 3 to 5 hr (Poceta, 2011). Automatisms (amnesia type episodes), are episodes that begin during the day, either from ingesting zolpidem

accidentally or on purpose. Parasomnias such as sleep-walking or sleep-driving occur when the dose is taken at bedtime. The cases reviewed in this study confirmed these types of complex behavior can occur after ingesting zolpidem. The case studies reflect the importance of knowing a patient's health history, as well as other medications being prescribed.

A thorough literature review was performed for the Poceta study. The author did identify the focus of the study and followed through with possible solutions and suggestions to avoid zolpidem-associated complex behaviors. This was both a qualitative and quantitative study. Statistics taken from the various cases brought in a quantitative side to the study; however, it was primarily a qualitative study reviewing human behavior. There was no mention regarding whether or not the subjects were advised they were a part of the study. The author did discuss the limitations of the study including a lack of information regarding the patients and defendants, a lack of medical history, and possible sleep disorders. A further limitation of the study was the unknown time of ingestion versus the time of the blood draw for analysis. Only three of the cases had a history of sleep studies being performed. Of the legal cases, all were trying to avoid DUI convictions which may have swayed their statements. Based on the types of impairment identified, these cases support the position of zolpidem being a drug which should be investigated with respect to aviation safety.

The second study, Logan & Couper (2001), was performed due to the number of subjects arrested for impaired driving and under the influence of zolpidem. The authors reviewed the literature on the pharmacology and performance effects of zolpidem and documented the impairment, driving behavior, and patterns of combined drug use to be employed to assist in the evaluation of future cases. Vertical gaze nystagmus is an involuntary up and down movement of the eyes and horizontal gaze nystagmus is an involuntary movement of the eyes from side to

side. Both are commonly used in field sobriety tests and were used in this study (Logan & Couper, 2001).

The subjects of the research were both male and female ranging in age from 16 to 77. The subjects were all arrested for impaired driving. Their cases were referred to the Washington State Toxicology Laboratory by law enforcement agencies for drug and alcohol testing. When the cases were received by the Laboratory and the alcohol concentration did not account for the subject's degree of impairment, the specimens were screened for other drugs. Those positive for zolpidem were used in the study.

When further analysis was necessary, the samples were tested by enzyme multiplied immunoassay technique (EMIT) and gas chromatography/mass spectrometry (GC/MS) for weak acidic or basic drugs. The EMIT screened for cocaine metabolites, opiates, amphetamines, cannabinoids, methadone, phencyclidine, propoxyphene, barbiturates, benzodiazepines, and tricyclic antidepressants. Zolpidem was located in the basic fraction of the drug screen. When zolpidem was identified, further data from the other drug and alcohol testing performed on the subject's specimens was obtained, as well as the arrest report. The samples were confirmed and quantitated for zolpidem by GC/MS using a five-point calibration curve from 0.00 to 1.00 mg/L. The linear range was 0.05 to 2.00 mg/L, the LOQ was 0.05 mg/L, and the correlation coefficient was typically better than 0.990.

A review of other studies provided peak plasma concentrations, time-to-peak and half-lives for 10 and 20 mg doses, respectively. This information was compared with the results of the cases in this study, as the time of the blood draw after driving was known, however, not the time of ingestion of the drug. The cases were reviewed in two categories. (1) Zolpidem was the

only drug present, and (2) other drugs were present as well. Five of the cases were only positive for zolpidem. Impairment signs included slow and slurred speech, slow reflexes, disorientation and confusion, lack of balance and coordination, loss of short-term memory, and "blacking out".

In the cases with other drugs present, the symptoms were mainly central nervous system (CNS) depression. Nine of the subjects were evaluated by drug recognition officers (DRE), five of the cases involved erratic driving with near collisions and the other four involved vehicle collisions. The signs of impairment were horizontal gaze nystagmus, vertical gaze nystagmus, lack of balance and unsteady gait, poor or slow coordination, poor performance of standardized field sobriety tests, slow and/or slurred speech, muscle flaccidity, and impaired or double vision. They appeared drowsy, tired, confused, and disoriented. The other drugs being present would have increased the impairment.

Logan & Couper concluded that zolpidem, even at normal doses, has the potential to affect driving negatively for several hours after ingestion. They also concluded that higher concentrations above the therapeutic range would further decrease a person's driving ability and level of consciousness. These conclusions support proceeding with the study of postmortem distribution of zolpidem for aviation safety. A drug having such impairing effects on a person's motor skills would definitely be a drug worthy of further investigation for aviation safety.

The authors performed a thorough literature review. They identified the focus of the study as the effect that zolpidem has on driving and what affect other drugs may cause when combined with zolpidem. This article did include a quantitative analysis of the drugs; however, the main focus was qualitative in respect to the effects of zolpidem in pulling information together through the literature review and case review to support the topic. The study made no

mention of limitations or weaknesses. However, one limitation in this study was the lack of knowledge of the time zolpidem was ingested to the time of the blood draw for analysis. Additional information on the subjects prior medical, prescriptions, and living arrangements would enhance the results of the study as certain medical conditions such as sleep apnea may add to the effects of the drug, and prescriptions often interact with each other causing increased symptoms. There was no mention whether the subjects were advised they were a part of the study.

Jones & Holmgren (2012) published a study regarding zolpidem and zopiclone concentrations from both impaired drivers and forensic autopsies. This article was selected for review due to the information pertaining to zolpidem and its impairing effects. The non-parametric Mann Whitney U Test was used. For the purposes of this article, non-parametric tests pertain to a test in which the data doesn't belong to a particular distribution, or it does not assume the structure of a model is fixed (Non-Parametric Test, n.d.) (no date). The Mann Whitney U Test is used here to assess if one of two samples of independent operations has a larger value then the other (Mann Whitney U Test, n.d.).

The purpose of this research was to compile a large number of forensic cases to assist toxicologists in interpreting concentrations of zolpidem and zopiclone in relation to the cause of death (Jones & Holmgren, 2012). This article was based on a study of positive findings of zolpidem and zopiclone from peripheral blood samples over a ten year period. It consisted of analyzing venous blood from living impaired drivers and femoral blood from autopsies. The autopsy cases were categorized into groups of intoxication or other causes. They were also categorized into multiple drug cases and those with only zolpidem or zopiclone present in the blood samples. The age range of the traffic offenders was 29-57 for zolpidem, and 28-52 for

zopiclone. The age range for autopsy cases was 38-76 for zolpidem, and 40-74 for zopiclone. The deaths from drug intoxication ranged from ages 38-70 for zolpidem, and ages 36-68 for zopiclone. Deaths from other causes ranged from age 42-76 for zolpidem, and age 43-77 for zopiclone. The subjects were both male and female.

All data was obtained from TOXBASE, the forensic science database, in Sweden (Jones & Holmgren, 2012). In 1999, Sweden began a zero tolerance law that resulted in an increase in the number of cases cited for driving under the influence of drugs (DUID). Any driver with an unusual appearance or behavior when questioned by the police, anyone showing signs or symptoms of being under the influence, dangerous driving, having a traffic accident, or moving traffic offense, would have a roadside breath-alcohol test and require specimens of blood and urine for analysis. The results from the analyses of these cases are all input into TOXBASE. All autopsy results were also entered into TOXBASE.

Toxicology testing was performed on the blood samples from both the autopsy cases and impaired driving subjects. An initial screening was completed by immunoassay either EMIT or cloned enzyme donor immunoassay (CEDIA), on the urine samples. If urine was not available, the blood samples would be screened after the precipitation of proteins. All positive cases were confirmed by either GC-MS or LC-MS with deuterated standards of the drugs used for the internal standard. Ethanol was confirmed by headspace GC. The extraction was liquid-liquid using n-butyl acetate followed with analysis by capillary column GC with a nitrogen-phosphorous detector.

TOXBASE was searched for positive zolpidem cases. Those cases were grouped into categories by International Classification of Diseases-9 codes (ICD-9 codes). The codes were

assigned to each case by the pathologists considering all circumstances such as the police report, witness statements, toxicology results, anatomy, police investigation and histology. If femoral blood was not available, the cases were omitted from the study. The cases were grouped into those from drug intoxication and other causes. TOXBASE was also reviewed for the DUID cases.

The statistical tests used to analyze the data were group comparisons by medians and non-parametric tests. Mean, median, standard deviation (SD) and upper percentiles were used. Student's independent t-test compared the mean age of the subjects. Two median concentrations were compared by the Mann-Whitney test. The differences between two means or two medians were considered significant if $p < 0.05$.

For the demographics, the traffic offenders were younger by an average of 15-20 years (range) compared to the subjects of autopsy cases. When the cause of death from the autopsy cases was drug intoxication, the subjects were younger by an average of 5-8 years (range) than those of autopsy cases. In the traffic cases, the number of males was greater than the number of females by a 75/25% margin. No gender difference was noted in the autopsy cases.

In the analytical portion of the study, the blood concentration of zolpidem and zopiclone was higher in the intoxication deaths compared to deaths from other causes. There was no significant difference in the traffic cases. Overall, when only zolpidem or zopiclone were detected, the concentrations of each were higher than in multi-drug cases. Zolpidem was detected more often and at a higher concentration than zopiclone. Zolpidem was the only drug detected in 28% of the driving offenders. Zopiclone was the only drug detected in 15% of the driving offenders. The concentration of zolpidem in cases with other drugs was 0.16-0.26 mg/L.

In cases with only zolpidem, the concentration median was 1.35 mg/L. The concentration of zopiclone in cases with other drugs was 0.05-0.11 mg/L. In cases with only zopiclone, the concentration median was 0.70 mg/L.

After eight hours of sleep, the concentration of zolpidem or zopiclone in blood should be low due to their short half-life. The findings of these drugs in the blood of drivers during all times of the day, suggests the drugs are either being improperly used or abused. This study showed intoxication deaths were higher when several drugs were ingested, suggesting the drug interactions may have contributed to the deaths. When combined with alcohol or benzodiazepines symptoms of toxicity, including performance impairment, increase for both zolpidem and zopiclone. Ethanol was found most prevalent in autopsy cases and diazepam in the motorists. The data from this study should be useful when interpreting concentrations of zolpidem or zopiclone in blood samples for the cause of death.

This article did include a thorough literature review. The limitations and/or weaknesses of this study are the lack of medical and personal history on the subjects, the lack of factual information on what drugs were taken and when, and the lack of information regarding the time interval between death and toxicological testing. The study was both quantitative and qualitative. It was quantitative in relation to the drug concentration data, yet qualitative with respect to drug effects. Given the data compiled, it proves both zolpidem and zopiclone are becoming highly used and abused drugs. The possibility of abuse of zolpidem, and its effects on driving ability noted from this article confirm the need for the proposed postmortem distribution of zolpidem study.

This portion of the literature review addressed three articles relating to the effects of zolpidem. All three articles did support the need for the proposed study. The first two articles were both researching the effects of zolpidem such as amnesia and sleep driving in Poceta's (2012) study and a variety of effects reported in Logan and Couper's study (2001). Jones and Holmgren (2012) took a different approach as they put together a compilation of autopsy and impaired driving cases which tested positive for zolpidem and zopiclone to assist in interpreting concentrations in future cases, in relation to the cause of death. These studies would be enhanced by the proposed study of the postmortem distribution of zolpidem.

Jones and Holmgren's (2012) study referenced that zolpidem concentrations from Logan and Couper's (2001) were in good agreement with their study. The mean concentration from the Logan and Couper (2001) study was 0.29 mg/L, and the median 0.19 mg/L. In the Jones and Holmgren (2012) study the mean concentration was 0.31 mg/L, and the median 0.19 mg/L. The mean concentration from the Poceta (2011) study was 0.17 mg/L, and the median was 0.11 mg/L. Poceta's (2011) was much lower; however, had fewer samples then the other two studies. The difference with the proposed postmortem distribution study would be including a lower concentration range and a greater number of specimens, to include body tissues.

The samples from all three studies were analyzed by GC-MS or LC-MS. The proposed study for postmortem distribution utilizes the UPLC-MS-MS, a relatively new instrument to the field of forensic toxicology. The results of all three instruments would be validated for reliability per industry standards.

This first section of literature review supported the need to study the postmortem distribution of zolpidem due to the side effects the drug can cause. The next section will review

three articles pertaining to research similar to that of the proposed postmortem distribution study in an effort to support the need for further study while avoiding duplication.

Has Postmortem Distribution of Zolpidem Been Researched?

Zolpidem is a non-benzodiazepine sedative-hypnotic with a short half-life of approximately 2 hr (Levine, Wu, & Smialek, 1999). It is metabolized with less than 1% of a dose appearing in urine as unchanged drug. After an oral dose, the bioavailability is approximately 70% (Levine et al., 1999). The following article describes a study investigating the distribution of this drug.

The purpose of Levine et al. (1999) was to study the tissue distribution of zolpidem in postmortem cases. The data for the study was collected from 8 cases at the Office of the Chief Medical Examiner in Baltimore, MD over a 3-year period. All of the cases had tested positive for zolpidem. There was no personal data or case history noted in the article other than the cause and manner of death. Of the 8 cases, 5 of the deaths were due to drug intoxication and the other 3 were not drug related.

All cases were screened for volatiles, therapeutic medications and drugs of abuse. Volatile testing included methanol, ethanol, acetone, and isopropanol by head space gas chromatography. Acid/neutral and alkaline testing was performed by gas chromatography with nitrogen-phosphorus detection (GC-NPD). Morphine was tested by radioimmunoassay and acetaminophen, ethchlorvynol, and salicylate by colorimetry.

This article did consider the distribution of zolpidem in postmortem cases, however, there were not any notable findings regarding any type of correlation between the blood and tissues. The notable finding was that the blood concentration was higher than urine, making blood the specimen of choice for analysis.

This study did provide additional information in the area of zolpidem distribution in postmortem cases. It was not, however, as extensive as the study outlined in this proposal in looking for a correlation between the blood and other specimens. The brain was not tested. The study indicated the brain is not usually collected for toxicological analysis. In the proposed study, the brain is an available specimen as it is routinely collected in aviation accidents. No limitations or weaknesses were mentioned in the article. The limited number of cases and specimen types being analyzed would be considered a limitation of the study. There were several citations from other literature showing concentrations of different cases. There was no comparison made between the results of the other studies and the Levine et al. (1999) study. The study was both quantitative and qualitative. The analytical study of concentrations was quantitative and the review of the distribution of the drug was qualitative.

The next article reviewed was a project similar to the proposed. The purpose of this project was to present data on the distribution of zolpidem in body fluids and organs from 5 fatal cases. The specimens included blood, urine, stomach contents, brain, lung, liver, and kidney (Takayasu, Ishida, Kimura, Kawaguchi, & Kondo, 2008).

The authors are from two Japanese Universities but no mention was made as to where this study was actually performed. Of the 5 cases used in this report, 3 subjects were female and 2 male. The ages were, a teenager, one in their 30's, two in their forty's and one subject in their

50's. One death was from hypothermia, 1 a drowning, and 3 were drug intoxications. All subject specimens were positive for zolpidem.

The urine samples were screened by Toxi-Lab® and Triage® systems. Toxi-lab® is a thin layer chromatogram drug identification system, performed by extracting the drug in an organic solvent and then separating by thin layer chromatography separation. The chromatogram is developed using various chemicals and after drying, the drugs are visually seen by a staining technique (Varcoe, 2001). Triage systems is a point of care assay requiring incubation of the sample with antibodies, transferring and washing the sample and the use of a dipstick for testing (Shaw et al., 2001). The blood and urine were analyzed for ethanol by headspace GC using tert-butanol as the internal standard. GC-MS was used to screen for basic drugs. For analysis, after solid phase extraction of positive samples, a model Trace GC-MS system was used in the positive electron-impact ionization mode.

In cases 2 and 4, the zolpidem concentration in the solid tissues was much higher than that in blood (the following results are listed by case 2, 4 respectively; 0.231 and 0.211 μg/g, blood, 1.55 and 3.63 μg/g in stomach contents, 0.733 and 0.500 μg/g in the brain, 0.554 and 0.322 μg/g in the liver, and 0.633 and 0.502 μg/g in the kidney). Case 3 had high values in all of the specimens, 1.04 μg/g blood, 1.91 μg/g urine, 76.8 μg/g stomach contents, 2.66 μg/g brain, 4.03 μg/g lung, 10.9 μg/g liver and 4.52 μg/g in the kidney. Since the zolpidem concentration was high in the stomach contents, it was considered that absorption was still occurring when the victim died. The zolpidem concentration in the liver was much higher than in any other solid tissues. One of the cases was considered at a lethal level and four of the cases were considered toxic. The study concluded that the levels of a drug in the organs are useful, as in cases in which blood is not available.

The article reported a distribution of zolpidem using high zolpidem concentrations. This article was the only one located similar to the proposed study. The differences are, first, that the proposed study anticipates including lower level concentrations and secondly, additional specimen types. The article set out to present data on the distribution of zolpidem from body fluids and tissues and was successful in doing so. There were no limitations mentioned in the article. The small number of cases studied is a limiting factor. The study did confirm the need for additional research such as the proposed of the postmortem distribution of zolpidem as it will increase the knowledge base with a greater range of concentration and specimen types. The study was both quantitative and qualitative in nature in reviewing the concentrations as well as the distribution of zolpidem.

The final article reviewed to find studies similar to that proposed, is a reporting of 2 zolpidem overdose cases. The purpose of this article was to describe 2 suicide cases of acute zolpidem overdose which included toxicological analysis of blood, vitreous humor, bile, urine, gastric contents, and liver, as well as the corresponding blood/vitreous humor ratios (Gock, Wong, Nuwayhid, Venuti, Kelley, Teggatz, & Jentzen, 1999).

The Gock et al. (1999) study took place at the Milwaukee County Medical Examiner's Office and Department of Pathology, housed at the Medical College of Wisconsin, in Milwaukee, Wisconsin. The subjects of this case were 2 victims, deceased from acute zolpidem overdose. Both were female, ages 36 and 58. Extensive case histories were given regarding the 2 victims in assisting the determination of the cause of death. The specimens analyzed included vitreous humor, gastric contents, urine, bile, liver, and blood.

Screening analysis was performed on both cases by an acid-dichromate test for volatiles, IL 482 Co-oximeter for carboxyhemoglobin, and color spot tests on urine for acetaminophen metabolites, ethchlorvynol, salicylates, and phenothiazines. Urine and gastric specimens were screened for acid, base, and neutral drugs by Toxi-Lab AB® thin-layer chromatography. Urine was screened by EMIT immunoassay for barbiturates, benzodiazepines, cannabinoids, and cocaine metabolite. GC was used to screen for zolpidem. The positive specimens were analyzed using an HP 5890 GC and equipped with an HP 5970 mass selective detector and an HP-1 capillary column.

The results of these studies included zolpidem concentration levels higher than that expected for therapeutic doses. According to Uges (2004), the therapeutic concentration for zolpidem is in the range of 0.08–0.15 mg/L. The toxic level is 0.5 mg/L and the lethal concentrations begin in the 2-4 mg/L range. The results were consistent with other published zolpidem fatalities. The concentration of zolpidem in the blood of case one was 4.5 mg/L in the subclavian blood and 7.7 mg/L in the iliac blood. Both of these concentrations fall within the lethal range. Case two, was 1.6 mg/L iliac blood, which was just below the suggested lethal range. In case 1, the subclavian blood was drawn within 24 hr after death. The iliac blood was drawn during the autopsy, around 24 hr after the collection of subclavian blood. The level increased; however, it was not representative of postmortem distribution of antidepressants (Gock, 1999). The high blood to vitreous humor ratios along with zolpidem being in the gastric contents, are indicative of acute zolpidem ingestion. The blood to vitreous ratio of the two cases was 2.81 and 3.08 for cases 1 and 2, respectively.

The conclusion of this article was the blood and vitreous humor ratios were not conclusive for determining the presence or absence of postmortem redistribution of zolpidem.

The cause of death in both cases was acute zolpidem overdose by suicide. The article did include numerous citations from their literature review. The limitation would be the small dataset; however, the study was for the purpose of reporting two cases, so it did meet this criterion. The report does lend more information to support the need for the proposed study. It was a mix of both quantitative and qualitative data. The study was quantitative in the aspect of determining the concentrations by analysis; and qualitative in the review for postmortem distribution.

All three articles reviewed for the postmortem distribution of zolpidem supported the need for the proposed study. It will not be duplication of efforts as there has not been a study that includes anticipated therapeutic concentrations, and in addition to the broad variety of specimens to be examined. The Takayasu et al. (2008) article was the only study located that was similar to the proposed work. The difference was the zolpidem concentrations and types of specimens analyzed. All other articles offered additional data, however, no specifics regarding distribution. There has not been sufficient data presented to make a correlation between the blood and tissue concentrations. The Levine (1999) study did discuss postmortem distribution; however, there was no attempt at blood/tissue correlations. The Gock et al. (1999) study discussed 2 cases with data and information which did include tissue specimens but not for any correlation between specimens. Now that both the first 2 sections of this literature review support the need for the study of postmortem distribution of zolpidem as proposed, the next section will look at the methodology to be considered for the study.

What Analytical Methodology Will Be Good For The Proposed Study?

This literature review has confirmed the need for the proposed research project on the postmortem distribution of zolpidem. A review of published analytical techniques and methodologies were necessary to determine what instrumentation would best suit the needs of the proposed study. There are several instruments which can be used to analyze zolpidem. Among them are as follows: GC-MS, HPLC, capillary electrophoreses (CE), LC-MS/MS, and UPLC-MS/MS (Shi, Xiang, Shen, B., & Shen, M., 2012). The three articles being reviewed in this section are all based on ultra-performance liquid chromatography (UPLC). This is the newest of the instruments so any articles using this technology would have knowledge of the older techniques and be able to make some type of comparison.

The first article being reviewed was based on a study in China whose concern was drug facilitated crime (DFC) cases. China has seen an increase in robberies and drug-facilitated sexual assault (DFSA) cases. Alcohol, cannabis, benzodiazepines, and hypnotics such as zolpidem are the drugs most commonly found in these cases. Zolpidem is now the preferred drug over benzodiazepines in treating short-term insomnia. As the Shi et al. (2012) article indicates zolpidem acts rapidly and due to the drugs amnesic properties, the victim is less likely to remember the offense that took place. For the purposes of this article, emulsification means breaking down into smaller particles. Protein precipitation is adding a salt or organic solvent to eliminate the contaminants found in blood.

The purpose of this study was to develop a rapid and accurate UPLC-MS/MS method for determination of zolpidem and its metabolites in biological fluids for forensic drug facilitated crime cases. The metabolites were included in this study to prolong the window of detection, as there is often a delay in reporting the zolpidem crimes due to the state of amnesia. UPLC-

MS/MS was the instrument used for this study because it was one of the most promising developments in fast chromatographic separations (Shi et al., 2012).

The authors are noted to be from the Shanghai Key Laboratory of Forensic Medicine, and the Shanghai Medical College at Fudan University, in Shanghai, China. The negative blood came from the Shanghai blood bank in China and the negative urine was donated by laboratory workers. There was a DFSA case discussed in the study, however, the article did not indicate where the case was from. The case was about a female; her case history was given with no other identifying information. Also noted in the study, were positive drug samples from one male and three female patients. No other identifying information for these samples was indicated.

There are several options for sample preparation. Liquid-liquid extraction (LLE), solid-phase extraction (SPE), and protein precipitation are the most common. Shi et al. (2012) indicates LLE is harmful to human health due to the large amount of organic solvent. They did not believe repeatability was satisfactory due to emulsification. SPE is more complicated being more costly and time consuming. Protein precipitation is easy and quick and uses less sample and organic solvent. Protein precipitation was selected for this study in an attempt to minimize the chances of errors, save time, and simplify sample preparation.

Samples were collected from the four patients at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 16.0, and 24.0 hr after the administration of a single oral dose of a 10 mg zolpidem tartrate. Urine samples were collected at 1, 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 80 hr after the dose was taken. To 10 ng zolpidem-d6, the internal standard used, 0.1 mL blood was added, 0.1 mL mobile phase (ammonium acetate and formic acid in water), and 0.8 mL acetonitrile. Acetonitrile was added to deprotonate the samples and remove impurities. The samples were

centrifuged at 10,000xg, for 3 min and then dried under a stream of nitrogen. The drug was reconstituted using 100 µL of methanol. Injection into the UPLC-MS/MS was 10 µL. The urine samples were prepared in the same way.

The analysis was performed on an Acquity UPLC purchased from Waters. The MDS Sciex API 4000 QTrap MS/MS, employed from Applied Biosystems. The column used, a Waters Acquity UPLC HSS T3. The total run time was 4 min operating on the ESI positive mode. Multiple reactions monitoring (MRM) mode was used for each analyte, monitoring for the parent ion and 2 daughters, for zolpidem, zolpidem d-6, the metabolites zolpidem 6-carboxylic acid (ZCA) and zolpidem phenyl-4-carboxylic acid (ZPCA).

The method was validated using spiked drug free blood and urine. The study evaluated the selectivity, stability, linearity, LOD, LOQ, matrix effect, recovery, intra-day, and inter-day precision and accuracy. The calibration curve was analyzed using least square weight ($1/X^2$) linear regression. LOD was selected when signal-to-noise was greater than 3, LOQ greater than 10. For the method to be accepted, the intra- and inter-day precision and accuracy had to be below 15%.

The results of the method validation was a calibration curve from 0.1-300 ng/mL in blood for zolpidem, 0.1-500 ng/mL for ZPCA, 0.1- 200 ng/mL for ZCA. The urine curves were 0.1 - 600 ng/mL for zolpidem and ZPCA, and 0.1-300 ng/mL for ZCA. The regression coefficient was higher than 0.9995. The LOD was 0.05 ng/mL and LOQ was 0.1 ng/mL, for all analytes. The intra- and inter-day precisions were less than 15%. Recoveries ranged from 70.0% to 98.3%. The matrix effect was between 79.5% and 99.0% and the process efficiency was between 60.7% and 94.4%.

At 16 hr post administration, zolpidem was not detectable in blood, 40 hr for urine. ZPCA was determined to be the major metabolite of zolpidem. It could be detected in blood up to 24 hr and urine for 72 hr. The peak concentrations occurred at 1.5 hr in blood and 8 hr in urine, after a single dose. Due to differences in other published times; Shi et al. (2012) believe there may be a different metabolic profile for the Chinese as opposed to Caucasians.

A DFSA case with samples collected 20 hr after the assault, were found to have 10.5 ng/mL ZPCA, but no zolpidem or ZCA in the blood. All three were detected in the urine at concentrations of 8.9 ng/mL for zolpidem, 94.2 ng/mL for ZPCA, and 13.6 ng/mL for ZCA.

The authors of this study were successful in developing and validating a rapid and accurate UPLC-MS/MS method for determination of zolpidem and its metabolites. The method would be suitable for the proposed study. The study quantitatively determined concentrations of samples, and qualitatively compared the methods and extractions with other available options. No limitations were mentioned in the article. No specific detail was noted as far as where the study took place. Numerous citations from other publications were identified in the article indicating a thorough literature review had been done.

Due to small doses and low therapeutic concentrations, it is important to use sensitive and reliable methods. With a multi-drug method, baseline separation of the analytes must be considered. Adjusting the gradient at the least amount of time minimizes the cost per sample and optimizes the sample throughput (Nielsen & Johansen, 2012). All of this was considered in the next article.

In a published study by Nielsen & Johansen (2012), they designed, developed, and validated a UPLC/MS/MS method multi-targeted for 25 pharmaceuticals in whole blood using a

LLE. The study, performed in Denmark, consisted of samples obtained from autopsies. Blood was collected from subjects suspected of criminal offenses. No additional information was provided regarding the participants in the study or the location where the study was performed.

The sample preparation included 0.10 g of blood added to 20.0 µL of 0.25 mg/L internal standard and 150.0 µL water. The samples were then extracted with butyl acetate after adjusting the pH with 2M NaOH. The samples were then vortexed and centrifuged. The supernatant was removed and evaporated to dryness under a stream of nitrogen. The samples were reconstituted in 100 µL mobile phase which included methanol, acetonitrile, and formic acid, injecting 5 µL into the UPLC.

A Waters Acquity UPLC was utilized with a tandem quadrupole, Acquity TQD mass spectrometer from Waters. Ionization ESI positive mode with data acquired in multiple reactions monitoring (MRM) mode. The samples were also run on HPLC-MS/MS using an Agilent 1100 with binary pump. Detection and quantification were performed using a Waters, Quattro Micro triple quadrupole mass spectrometer. Ionization was also ESI positive and acquisition in MRM mode

The UPLC-MS/MS method validation was issued by the Danish Accreditation and Metrology Fund with small deviations. The parameters evaluated were LOD, LOQ, recovery, selectivity, carryover, linearity, accuracy, and precision.

In a comparison of the HPLC-MS/MS to the UPLC-MS/MS, ion suppression was noted for 2 of the drugs by HPLC. N-demethyl-clozapine was suppressed 40% by zolpidem which eluted close to it. Promethazine was suppressed 50% by orphenadrine when at a high concentration. On the UPLC-MS/MS, there was a good baseline separation between them. On

the UPLC-MS/MS, the ion suppression noted was levomepromazine at 30% by amitriptyline. Quantification can be performed using another gradient for this.

For the matrix effect, recovery and carryover, a matrix effect value above 0 indicated ion suppression and a value below 0 indicated ion enhancement. Both mirtazapine and zuclopenthixol had 13% ion suppression. All other analytes were within 3%. The coefficients of variation were below 4%, with the exception of mirtazapine which was 16%. The recovery was between 55 and 87% for all analytes except norfluoxetine which was 44%. Carryover was less than 0.3% for all analytes and internal standards. The LOD was three times the standard deviation. They ranged from 0.0005 to 0.0082 mg/kg. The LOQ for most compounds was 0.002 mg/kg. The linearity was in the calibration range of 0.002 to 4.0 mg/kg. Most of the analytes were in the range 0.002 to 2.0 mg/kg with r^2 greater than 0.99, except norfluoxetine, which was 0.9862. 1/x weighting was used. Lamotrigine and lidocaine were prepared directly and diluted with water. A 1 point calibration curve was used for daily analysis. The 1 point was forced through zero. The coefficient of variation (CV) and BIAS (accuracy) were also performed with a 1 point calibration curve. The intra-day and inter-day precision was below 20% at levels above the LOQ for all analytes except mirtazapine which was 33% and zuclopenthixol at 27%. The accuracy was within 20% except levomepromazine at 29%, methylphenidate at 25%, norfluoxetine at 48% and zuclopenthixol at 25%.

This study was successful in developing a quantitative method with LLE and UPLC-MS/MS analysis for 25 common pharmaceuticals. No limitations were mentioned in the paper. The method development of the study was qualitative, and the analysis of the various analytes and study validation was quantitative. There was no information regarding where the study was

performed, or if the participants were notified of the study. This article does support the proposed study for the use of the UPLC-MS/MS, as well as, the sample preparation method.

In the final article of this section, the UPLC library will be considered. According to Humbert et al. (2010), GCMS has been the most used technique for screening of xenobiotics. Spectra obtained from the specimens are matched with those from large libraries of spectra formulated by using reference compounds. The use of LCMS has many advantages; however, the disadvantage is the lack of a universal library. The libraries vary according to the instrument manufacturer due to variation in spectra.

The purpose of this study was to build a new mass spectra library starting with 500 compounds for UPLC. The study was extended to evaluate transferring data from 5 collaborating laboratories to see if it was feasible to share the library, as well as the task of expanding it (Humbert et al., 2010).

The article does not specifically indicate where the study was performed, however, the authors are from France. The samples were extracted from patients and analyzed on the date of admission to the hospital. The name of the hospital was not indicated.

To prepare the working standards, the drugs being used were diluted 1:50 with methanol. Twenty µL of the working standards were mixed with 100 µL of IS. They were mixed, then evaporated under nitrogen, and reconstituted with 100 µL of mobile phase A which was a combination of formate buffer with formic acid. The final mixture was 60 µL of the drug and 150 ng of IS, 15 µL was analyzed, one in ES+ and one in ES-, both at 6 cone voltages. The ES± are electrospray ionization, a soft ionization technique with little fragmentation which produces ions. For each compound, 12 different spectra could be available to add to the library.

The biological samples were prepared by an LLE, both acidic and basic. For the acidic extraction, 1 mL of the sample, 100 μL of IS, and 500 uL sodium acetate buffer were added to a 10 mL tube and mixed. They were extracted by adding 3 mL of a mixture of dichloromethane, ether, hexane, and isoamyl alcohol. The samples were then vortexed for 2 min and centrifuged for 5 min. The upper organic layer was transferred to a clean vial. For the basic extraction, this was prepared as the acidic extraction; however, 500 μL of saturated disodium tetraborate solution was added in the first step instead of the sodium acetate buffer. The supernatant of each was evaporated using a vacuum concentrator and reconstituted in 100 μL of mobile phase.

The Acquity UPLC from Waters was utilized in this study. The column used, an Acquity UPLC HSS C18. Mobile phase A, formate buffer with formic acid; and mobile phase B, acetonitrile with formic acid. The injections were 15 μL. For the mass spectra a Waters TQ Detector tandem-quadrupole MS with Z-Spray source and ES probe was used. MassLynx controlled the instruments. Ionization was in ± ES. Data was acquired in full scan mode from 80 to 650 m/z. There were six different cone voltages for ES+ and 6 for ES-. Increasing the cone voltage fragments the molecule and the molecular ion disappears as cited in Humbert et al. (2010). The total run time was 15 min.

ChromaLynx was the application manager by Waters which was used to process the data. It automatically integrated the component peaks at each cone voltage. A deconvolution technique removed any contamination or non-specific background ions. This made a clean spectrum for the library.

The reproducibility of the retention times were studied over a 34 day period and found to be highly reproducible. The transferability of spectral information for the library was reviewed

and found successful with the 5 laboratories sampling a mixture containing 11 analytes. With so many drugs being tested, a "match factor" was used. All of the samples tested by the 5 laboratories had a match factor greater than 700. If the match factor was lower, there would be co-elution with overlaid chromatograms; it would not be possible to determine which ion was related to which peak. All analytes would be located, but would have lower match factors with the ions from other analytes lowering the overall match.

All samples were also analyzed using EMIT for immunoassay for screening of the barbiturates, benzodiazepines, and tricyclic antidepressants. HPLC-Photo Diode Array (PDA) was also used with the same extraction procedure. The benzodiazepines were detected by immunoassay. They were missed consistently by HPLC-PDA, but the UPLC-MS/MS confirmed the immunoassay result and also identified the specific compound and its metabolites.

UPLC columns have a small particle size. This allows better chromatographic resolution. Peaks are more intense and the risk for co-elution is lower. Of the 500 drug standards, 29 were in the negative ionization mode, 415 were in the positive ionization mode and 56 were in both modes. A total of 2975 spectra were produced for the library. The UPLC method was successful in identifying more analytes with more specific results than the immunoassay and the HPLC-PDA.

This article reflects the study was successful in building a mass spectra library with sharing ability. There were no limitations mentioned in the article. The limitations and/or weaknesses were that no specific information was mentioned as to where the study took place, and whether or not the participants were aware of the study. It was a quantitative study in the quantitation of analytes and the match factors obtained. Building the library put a qualitative

aspect into the study. Several citations were included in the article which assisted in confirming the reasoning for using the UPLC method. This also supplies additional confirmation for the proposed study to be performed by UPLC-MS. The extraction method is lengthier then others researched so probably would not be considered for the proposed postmortem distribution of zolpidem study.

The three articles reviewed confirm UPLC-MS would be a good instrument to be used in the proposed study. The extraction methods did vary with that being the biggest difference in the three articles. The first article described a protein precipitation method. The second and third articles both described LLE's. The first article discussed the different extraction methods and the protein precipitation method appears to be the best method for time, ease, and cost if it can be performed to obtain the desired results. All three articles did reach the end result anticipated for their studies. They all lacked information regarding the actual study as far as the location. Additional information as to where the samples came from would also have been of interest.

Literature Review Summary

After completion of the literature review, all nine articles reviewed support the proposed study on the postmortem distribution of zolpidem in aviation accident victims. There were numerous articles reviewed for this project. The nine used for this literature review were the most relevant for the area of study.

In the interest of aviation safety, pilots must be fit to fly the aircraft safely. Monitoring prescription medication and their effects is imperative for such safety. Before proceeding with

the proposed study, it first had to be determined if zolpidem could adversely affect aviation safety, thereby, suggesting that postmortem distribution research was necessary.

In the Poceta (2011) article, automatisms and parasomnias were discussed as effects of zolpidem. Automatisms are a period of amnesia when the drug is taken during the day. Parasomnias occur when the drug is taken at night such as in sleep-walking or sleep-driving.

In the second article reviewed, Logan and Couper (2001), discussed subjects who had been arrested for impaired driving and were positive for zolpidem in their systems. The signs of impairment were numerous including disorientation and confusion, slow and slurred speech, lack of balance and coordination, and "blacking out". All of these signs of impairment are reason to further study zolpidem.

In the last article reviewing whether or not there was a need to study zolpidem in relation to airline safety, Jones and Holmgren (2012) studied zolpidem and zopiclone in compiling cases to assist in interpreting concentrations in relation to the cause of death. In doing so, numerous cases were from DUID with positive findings for the two drugs. Their study indicated it appeared the drugs were either being improperly used or abused due to finding the drug in the blood of drivers during all times of the day. The autopsy cases showed intoxication deaths were higher when several drugs were ingested suggesting the drug interactions may have contributed to the deaths. Due to the results of this article, it concurred with the other two that zolpidem should be further studied in relation to aviation safety.

A lack of personal and medical information regarding the subjects was a limitation shared by all three articles. There was also a lack of information between the time of ingestion and time

of collection of the blood or specimens. Working on these areas of limitations would increase the validity of the studies.

The second section of the literature review was to determine what type of research had been performed in the area of postmortem distribution of zolpidem. Three articles were reviewed and all did support the need for the proposed study.

In the first article, Levine et al. (1999) studied the distribution of zolpidem. Eight cases were used in this study. The concentration of zolpidem in blood was higher than that in urine indicating blood would be the specimen of choice. No substantial findings were noted regarding the distribution of the tissues; however, liver and kidney were the only two specimens in the study. This article supports the need for the proposed study as the proposed will include additional specimens.

The second article was the most similar study to the proposed. Takayasu, et al. (2008) did find the levels of a drug in the organs are useful to cases in which blood was not available. The study was based on higher concentration cases. The proposed study anticipates including lower therapeutic concentration cases, as well as a broad array of specimen types.

In the final article of this section, Gock (1999) performed a review of 2 zolpidem suicide cases. One of the cases had a high zolpidem blood to vitreous humor ratio and zolpidem in the gastric contents, both pointed to acute zolpidem ingestion which lends itself to additional information for the distribution of zolpidem.

Taking into consideration the information gleaned from all three articles, there is still a significant gap to be filled regarding the postmortem distribution of zolpidem. This gap supports the need for the proposed study. Lack of data, or limited cases and/or specimens is a shared

limitation for all three articles. As in the first section, any increase in this area would also increase the internal validity of the studies.

The third section of the literature review compared the potential methods available to perform the proposed study. The purpose of the Shi et al. (2012) study was to develop a rapid and accurate UPLC-MS/MS method for zolpidem and its metabolites. The sample preparation was protein precipitation which proved to be quick and easy. The study was successful in method development and extended the window of detection by including the metabolite ZPCA.

The second article, a study by Nielsen et al. (2012), set out to develop and validate a multi-drug method for 25 pharmaceuticals using a LLE. The article supported the UPLC-MS/MS for the analysis of zolpidem. With the HPLC-MS/MS, another drug, n-demethyl-clozapine was suppressed 40% by zolpidem as they eluted very close to each other. Using the UPLC-MS/MS, there was a good baseline separation between them.

Lastly, the article by Humbert et al. (2010), set out to build a new mass spectra library starting with 500 compounds for the UPLC. This was a successful project and very useful for the UPLC-MS/MS field as one of the drawbacks of the instrument was the lack of a universal library. Five other laboratories took part in the study and they were successful in collaborating with Humbert et al. (2010) proving they can work together to build the library and share their findings.

From the literature reviewed, UPLC-MS/MS appeared to be the most innovative instrument to perform the postmortem distribution study, with the protein precipitation sample preparation. Again, all three of the articles shared the same limitation, lacking information regarding the location of the studies.

In general, none of the articles were specific regarding details of the studies such as where or how the studies were set up. None mentioned any consideration to ethics regarding the participants in the studies.

The nine articles included in this literature review did support the need for the proposed study of postmortem distribution of zolpidem in relation to aviation accidents. There are numerous physical and psychological impairments associated with ingestion of the drug. Additional research in the area is necessary as the research available is not sufficient to make any interpretation of concentrations of blood based on the tissues or body fluids. The UPLC-MS/MS does appear to be the instrument of choice, with a protein precipitation extraction for the study as it has proved to be successful in analyzing zolpidem in an efficient, rapid, reliable method.

Chapter III. Methods

Introduction

The lack of available blood in approximately 30% of victims of aviation accidents makes it difficult to interpret the concentration of drugs found in other available specimens. Blood or plasma is generally used to aid in the interpretation of drug concentrations from the toxicological analyses. Finding a way to interpret the concentration of a drug without a blood specimen was the issue being addressed with this research project.

There were two research questions to be answered in this study. Was there a consistent and reproducible postmortem distribution of zolpidem between one or more unique body tissues and/or fluids? If so, would it be possible to estimate zolpidem blood concentrations from zolpidem fluid and/or tissue concentrations?

Toxicological analysis was conducted on various specimens from cases that had previously screened positive for zolpidem at the FAA's Bioaeronautical Sciences Research Laboratory. The specimens were analyzed by UPLC-MS. The results obtained from the analysis were then reviewed for answers to this project's research questions. A new instrument to the Laboratory was being used for this research. Therefore, it was necessary to develop a method with the ability to analyze postmortem specimens.

This study was quantitative with some qualitative aspects. The portion of the study analyzing the calibrators, controls, and specimens resulting in concentrations was quantitative. The evaluation of zolpidem distribution and method development provided a qualitative aspect to the study.

Setting

This research project was completed at the FAA's Bioaeronautical Sciences Research Laboratory at the Civil Aerospace Medical Institute in Oklahoma City, Oklahoma.

Sample/Participants

The samples used for this experiment were nonrandom, purposive samples. The following criteria were used for selecting the cases. The first criterion was that samples would come from cases that have previously tested positive for zolpidem by the FAA. The second criterion was the case must contain a majority of the specimens desired for the research (blood, urine, vitreous humor, liver, lung, kidney, spleen, muscle, brain, and heart). All participants were deceased victims. The demographics of this study are limited; no information about the participants such as name, address, age, or any other personal information was made known for the purpose of participant anonymity.

Chemicals and Reagents

Zolpidem and zolpidem d7 were purchased from Cerilliant (Cerilliant Corp., Round Rock, TX) at concentrations of 1.00 mg/mL in methanol and 100 µg/mL in methanol, respectively. Sodium fluoride 99% A.C.S. was purchased from Sigma-Aldrich (Sigma-Aldrich; St. Louis, MO). UPLC grade water, UPLC grade acetonitrile, and Formic Acid Optima LC/MS were purchased from Fisher Scientific (Fisher Scientific, Inc.; Pittsburgh, PA). Double deionized water (DDW) was generated using an ELGA PURELAB Ultra water system (ELGA,

Inc.; Lowell, MA). Bovine blood was obtained from Country Home Meat Co. (Country Home Meat Co.; Edmond, OK).

A 1.0% NaF (sodium fluoride) solution was prepared by mixing 5 g NaF and 500 mL DDW in a 500 mL plastic squeeze bottle. All weights were measured on a Denver Instrument TL2102 Toploader balance (Denver Instruments; Bohemia, NY). UPLC grade acetonitrile was mixed with formic acid in a 999:1 (v:v) ratio, respectively, for mobile phase A. Mobile phase B was prepared with UPLC grade H_2O and formic acid in a 999:1 (v:v) ratio, respectively. A UPLC wash solution was prepared at an 80:20 (v:v) ratio with UPLC grade water and UPLC grade acetonitrile.

Sample Selection and Storage

A search of the CAMI toxicology database (ToxFLO^{TM}, DiscoverSoft Development, LLC; Oklahoma City, OK) identified 10 zolpidem-positive fatalities from separate accidents that occurred during a period of 4 years (2007-2010). Each of these cases had a majority of the desired biological tissues and fluids available for analysis. The blood from the cases was stored in tubes containing 1.00% (w/v) sodium fluoride/ potassium oxalate. In all cases, all specimens were stored at -20ºC prior to analysis.

Instrumentation and UPLC-MS Method

All analyses were performed utilizing a Waters® Xevo® TQ-S Acquity UPLC® system. The system includes an FTN auto sampler, binary solvent manager, and step wave ion transfer technology which increases the efficiency of ion transfer from the ion source to the quadrupole MS analyzer. The mass spectrometer portion of the LCMS was optimized for zolpidem using the Waters Intellistart™technology. Intellistart calibrates the mass, sets resolution, provides optimum tuning for samples, optimizes the API ionization source conditions for analytical needs, and monitors instrument parameters. The source temperature was set at 150ºC, desolvation temperature at 500ºC, and the desolvation gas flow at 1,000 L/hr which should optimize desolvation and sensitivity. Initially, Intellistart evaluated the applicability of 4 modes of compound ionization (APCI ± and ESI ±) and determined that +ES mode would provide maximum ionization. Intellistart then collected the precursor (parent) ion and evaluated numerous parameters to maximize the sensitivity/intensity for the product (daughter) ions (see Table 1 for zolpidem and Table 2 for zolpidem d7).

Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 column (2.1 x 50-mm, 1.7-µm). The UPLC system and column were obtained from Waters (Waters Corporation; Milford, MA). The UPLC was operated with a gradient from 70:30 mobile phase (B - water with 0.1% formic acid:A - acetonitrile with 0.1% formic acid) to 35:65 (B:A) at 2.8 min, 5:95 (B:A) at 2.85 min, and back to70:30 (B:A) at 3.88 min, holding until 4.5 min. The run time was 4.5 min. Flow rate was 0.400 mL/min. The binary solvent manager was checked to assure the psi was <12000 and the delta was <50 at the flow rate, delta below 50 indicating that the column is ready for use. The sample injection volume was 2 µL. The UPLC was equilibrated for approximately 30 min prior to use. Following use, the 70:30 (B:A) mobile phase

flowed through the column for 10 min. The column was then washed and stored in a 20:80 mixture of methanol:H_2O.

Materials

The materials used for this research were disposable beakers, 16 x 100-mm silanized round bottom tubes with caps, 16 x 100-mm silanized screw top tubes with caps, LC vials with caps and silanized inserts, 10 mL, 25 mL and 50 mL volumetric flasks with tops, 5 mL adjustable pipette, 1 mL adjustable pipette, 200 µL pipette, appropriate pipette tips, test tube racks, disposable plastic transfer pipettes, homogenizing bottles, Parafilm, Kimwipes, a sharpie marker, and deionized water.

Calibrator and Control Preparation

A calibration curve for zolpidem was prepared by serial dilution, utilizing bovine whole blood as the diluent. Calibrators were prepared from a 10 µg/mL zolpidem calibrator working standard. Controls were prepared in a similar manner as calibrators using a 10 µg/mL zolpidem control working standard. The working standards were prepared by diluting 100 µL of a 1 mg/mL methanolic zolpidem drug standard with DDW to 10 mL in volumetric flasks using a different manufacture's lots for calibrators and controls. Cerilliant lot number FE051310-01 was used for the calibrator working standard and Cerilliant lot number FE071811-02 was used for the control working standard. A 100 ng/mL working internal standard was prepared by pipetting 50

µL of 100 µg/mL methanolic zolpidem d7 drug standard into a 50 mL volumetric flask and diluting it to volume with DDW. Cerilliant lot number FC101811-01 was used.

A calibration curve was prepared at concentrations ranging from 0.2 to 800 ng/mL. A minimum of 7 calibrators were used to construct each calibration curve. Controls were prepared at concentrations of 1, 10, 100, and 500 ng/mL and analyzed with each batch of unknowns to verify the accuracy of the established calibration curve. The calibrators were prepared by serial dilution. The 800 ng/mL calibrator was prepared by pipetting 0.8 mL of the 10 µg/mL calibrator working standard into a 10 mL volumetric flask, filling to the mark with whole bovine blood. The flask was capped and mixed well. Serial dilutions were prepared by pipetting 5 mL of the 800 ng/mL calibrator to 5 mL whole bovine blood in a test tube to make the 400 ng/mL, continuing the dilution to 0.2 ng/mL.

The 500 ng/mL control was prepared by pipetting 1.25 mL of the 10 µg/mL control working standard into a 25 mL volumetric flask. The flask was diluted to the mark with whole bovine blood, capped, and mixed. The 100 ng/mL blood control was prepared by pipetting 0.25 mL of the 10 µg/mL control working standard into a 25 mL volumetric flask and diluting to the mark with whole bovine blood. After capping and mixing, the 100 ng/mL blood control was used to prepare the 10 ng/mL blood control by pipetting 2.5 mL of the 100 ng/mL blood control into a 25 mL volumetric flask and filling the flask to the mark with whole bovine blood. After capping and mixing, the 10 ng/mL blood control was used to prepare a 1 ng/mL blood control by pipetting 2.5 mL of the 10 ng/mL blood control into a 25 mL volumetric flask and filling to the mark with whole bovine blood, capping, and mixing. A negative control was prepared using negative whole bovine blood.

Sample Preparation and Extraction Procedure

Calibrators, controls and specimens were prepared by the following procedure. Tissue samples were prepared for homogenization by adding 1.00% NaF solution to the tissue sample in a 2:1 w:w (1% NaF solution:tissue) addition. Tissue samples were homogenized using an OMNI post-mounted mixer homogenizer (Omni International; Kennesaw, GA). To individual 16 x 100-mm screw-top tubes, 0.1 mL aliquots of each calibrator, control, postmortem fluid, and 0.3 g aliquots of each tissue homogenate (0.1 g wet tissue) were transferred. To each tube 0.1 mL of 100 ng/mL internal standard was added (10 ng total). The tubes were vortexed and 1.00 mL of cold acetonitrile was added to each tube, capped, and thoroughly mixed. The tubes were then centrifuged at 1500xg for 10 min in a Thermo Jouan C4i Centrifuge (Thermo Electron Corp.; Chateau-Gontier, France). Centrifugation removed the proteins and cellular debris from the samples. Once centrifuged, 0.2 mL of the supernatant from each tube was transferred to 16 x 100-mm round bottom tubes and placed in the TurboVap LV concentration workstation (Caliper Life Sciences; Hopkinton, MA), set at 40ºC and 15 psi, and evaporated to dryness under a stream of nitrogen. Once dried, the residue was reconstituted in 1 mL of 70:30% UPLC grade water:acetonitrile, and 0.1 mL of the reconstituted sample was transferred to LC vials with inserts. The vials were capped and transferred to the auto sampler.

Data Analysis

The calibration curve was prepared by plotting a linear regression of the analyte/internal standard response factor versus the analyte concentration for the calibrators and was used to determine the concentrations of zolpidem in the controls and specimens. The calibration curve was also used to determine the LDR, LOD, and LOQ (Figure 2 & Table 3).

The FAA's Bioaeronautical Sciences Research Laboratory has 3 criteria that must be met before a drug is reported as positive in a sample using a LC-MS/MS instrument. An analyte must have a minimum signal-to-noise ratio (S/N) of 10, a retention time ± 2% of the average calibrator retention time, and must have a product ion ratio ± 20% of the average calibrator product ion ratio (MS-MS spectral "fingerprint" identification). The LDR is the concentration range in which the calibration curve remains linear. The LOD is the lowest quantity of a substance that meets the identification criterion. The LOQ meets the same criteria as the LOD, plus has an experimentally determined value within ± 20% of its prepared concentration (Table 3).

Accuracy and precision was determined for the method. Accuracy is how close the measured value is to the target value, and is expressed as the relative error. Precision is the reproducibility of a measurement, and is measured by the CV or relative standard deviation for the experimentally determined concentrations.

Intra-day accuracy and precision were determined on day 1 of the experiment by extracting and analyzing 5 replicates of each control concentration along with a calibration curve. The mean, relative error, and relative standard deviation were evaluated from the data obtained on day 1. The controls were stored at 4ºC for extraction on days 2, 3, 7, and 43.

Inter-day accuracy and precision were evaluated by extracting 5 replicates of each of the 4 control concentrations (originally prepared on day 1) on days 2, 3, 7 and 43. Quantitative values were generated using the calibration curve prepared on day 1. The mean, relative error and relative standard deviation were determined from the data and would reflect the accuracy and precision of the method over a 7-day period (Table 4). The stability of zolpidem in whole blood was determined by evaluating the control concentrations determined on day 7 of the inter-day experiment and day 43 (Table 4).

Extraction Efficiency

Extraction efficiency (recovery) was determined by preparing 2 sets of samples, one set spiked with zolpidem prior to extraction and the other set spiked after extraction. The first set of samples was made up at 3 different concentrations, 1 ng/mL, 100 ng/mL, and 500 ng/mL. Enough was prepared so that 5 replicas of each concentration could be analyzed. The samples were prepared in the same manner as described in the calibration and control preparation section of this chapter. The second set of samples consisted of 15 tubes containing negative bovine blood. Five replicates of each concentration and 15 negative bloods were "extracted" as described earlier. From each sample 0.2 mL of acetonitrile supernatant was transferred to clean 16 x 100-mm round bottom tubes and the negative samples were spiked as follows. A 10 µg/mL zolpidem standard was prepared by pipetting 0.1 mL of 1 mg/mL zolpidem into a 10 mL volumetric flask. The flask was then filled to the mark with UPLC grade acetonitrile and mixed. The 10 µg/mL zolpidem standard was then used to prepare a 33.4 ng/mL zolpidem standard by pipetting 0.167 mL of the 10 µg/mL standard into a 50 mL volumetric flask. The flask was then

filled to the mark with UPLC grade acetonitrile and mixed. To evaluate the recovery of the 100 ng/mL control samples, 0.05 mL of the 33.4 ng/mL zolpidem standard was pipetted into 5 of the negative tubes, and 0.25 mL of the 33.4 ng/mL zolpidem standard was pipetted into 5 of the negative tubes to evaluate the recovery of the 500 ng/mL control samples. To evaluate the recovery of the 1 ng/mL control samples, 0.1 mL of the 33.4 ng/mL zolpidem standard was pipetted into a 10 mL volumetric flask and filled to the mark with acetonitrile to make a 0.334 ng/mL zolpidem standard. From the 0.334 ng/mL zolpidem standard, 0.05 mL was pipetted into the 5 remaining negative tubes. The spiked samples were then processed just like the positive samples. The spiked samples are considered 100% recovery of the drug.

The reasoning for the extraction efficiency being determined in this manner was derived by taking the amount of supernatant being dried down (0.2 mL), dividing by the total amount of the sample prepared (0.2 mL sample and 1 mL acetonitrile), the volume being transferred was 16.7% (0.2 mL/1.2 mL). The 0.2 mL supernatant of the negative bovine blood samples were then spiked with 16.7% of the control concentration amount. The average response factor from the positive group was divided by the average response factor obtained from the spiked group, and was multiplied by 100 to obtain the percent recovery for zolpidem at each control concentration (Table 3).

Matrix Effect

The evaluation of ion suppression was conducted by preparing 2 sample types at the same concentration, one in acetonitrile and the other made up in whole bovine blood. Three acetonitrile samples (solvent samples), and 3 bovine blood samples (matrix samples) were analyzed, each sample having the internal standard added. The "matrix factors" function in the Water's software program averaged the 3 solvent samples and the 3 matrix samples and determined the matrix effects by determining the difference between the 2 averaged results (Table 5).

Chapter IV. Results

In this research project, postmortem samples from zolpidem-positive cases were analyzed by UPLC-MS to evaluate if there was a consistent and reproducible postmortem distribution of zolpidem between one or more unique body tissues and/or fluids. And if there was, would it be plausible to estimate zolpidem blood concentrations from an alternative matrix in cases where there is no blood available, as an estimate of the blood concentration is necessary to determine ante mortem effects.

The MS portion of the LC-MS was initially optimized for zolpidem using Water's Intellistart. The parameters optimized included mass, cone voltage, collision energy, ion mode, and the precursor and product ions. The results from this report are indicated in Tables 1 and 2. For quantitative analysis, the precursor ion was 308.2 for zolpidem. The selected product ions were 263.1 and 235.3. For zolpidem d7, 315.1 was the precursor ion for quantitation purposes. The product ions selected were 270.2 and 242.0.

The LC was manually optimized by experimenting with different flow rates and mobile phase gradients. The optimized gradient was from 70:30 mobile phase (B - water with 0.1% formic acid:A - acetonitrile with 0.1% formic acid) to 35:65 (B:A) at 2.8 min, 5:95 (B:A) at 2.85 min, and back to70:30 (B:A) at 3.88 min, holding until 4.5 min. The flow rate was 0.40 mL/min. Sample injection volume was 2 µL. Zolpidem and zolpidem d7 had retention times of 0.92 min and 0.91 min respectively. The total run time was set for 4.5 min using + electrospray ionization.

Table 1

Intellistart Results for Zolpidem (307.4 m/z)

Precursor Ions (m/z)	Cone Voltage (V)	Product Ions (m/z)	Collision Energy (CE)	Ion Mode
308.2	2	235.3	32	ES+
308.2	2	220.0	44	ES+
308.2	2	248.1	32	ES+
308.2	2	205.2	52	ES+

Table 2

Intellistart Results for Zolpidem d7 (314.4 m/z)

Precursor Ions (m/z)	Cone Voltage (V)	Product Ions (m/z)	Collision Energy (CE)	Ion Mode
315.1	42	242.0	38	ES+
315.1	42	270.2	26	ES+
315.1	42	224.9	38	ES+
315.1	42	252.1	34	ES+

A calibration curve was constructed from 0.2 to 800 ng/mL for zolpidem in bovine blood. The correlation coefficient was 0.9999 with a weighting factor of 1/x. The curve was linear and excluded the origin. Thirteen points were used for the calibration curve (see Figure 2). The chromatogram illustrated in Figure 3, is the 800 calibrator from day 1. The top row is zolpidem

d7 and the bottom row is zolpidem. The chromatogram reflects a screen shot of each ion being captured as selected during method development. The retention time is also noted at the top of each peak. Table 3 lists the LDR, LOD, and LOQ. The extraction efficiency (% recovery) was determined at 1, 100, and 500 ng/mL and is listed in Table 3.

Figure 2

Zolpidem Calibration Curve

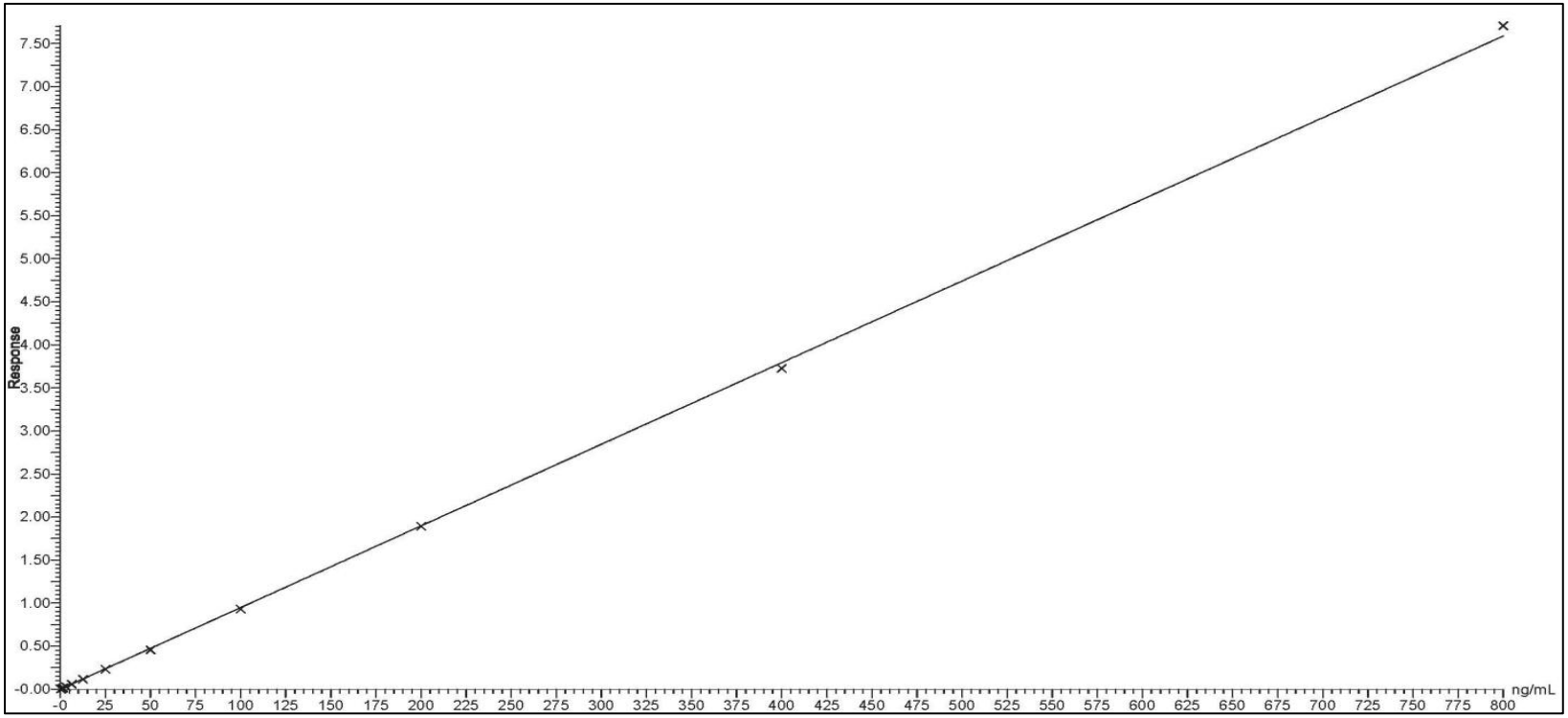

Figure 3

Zolpidem Chromatogram

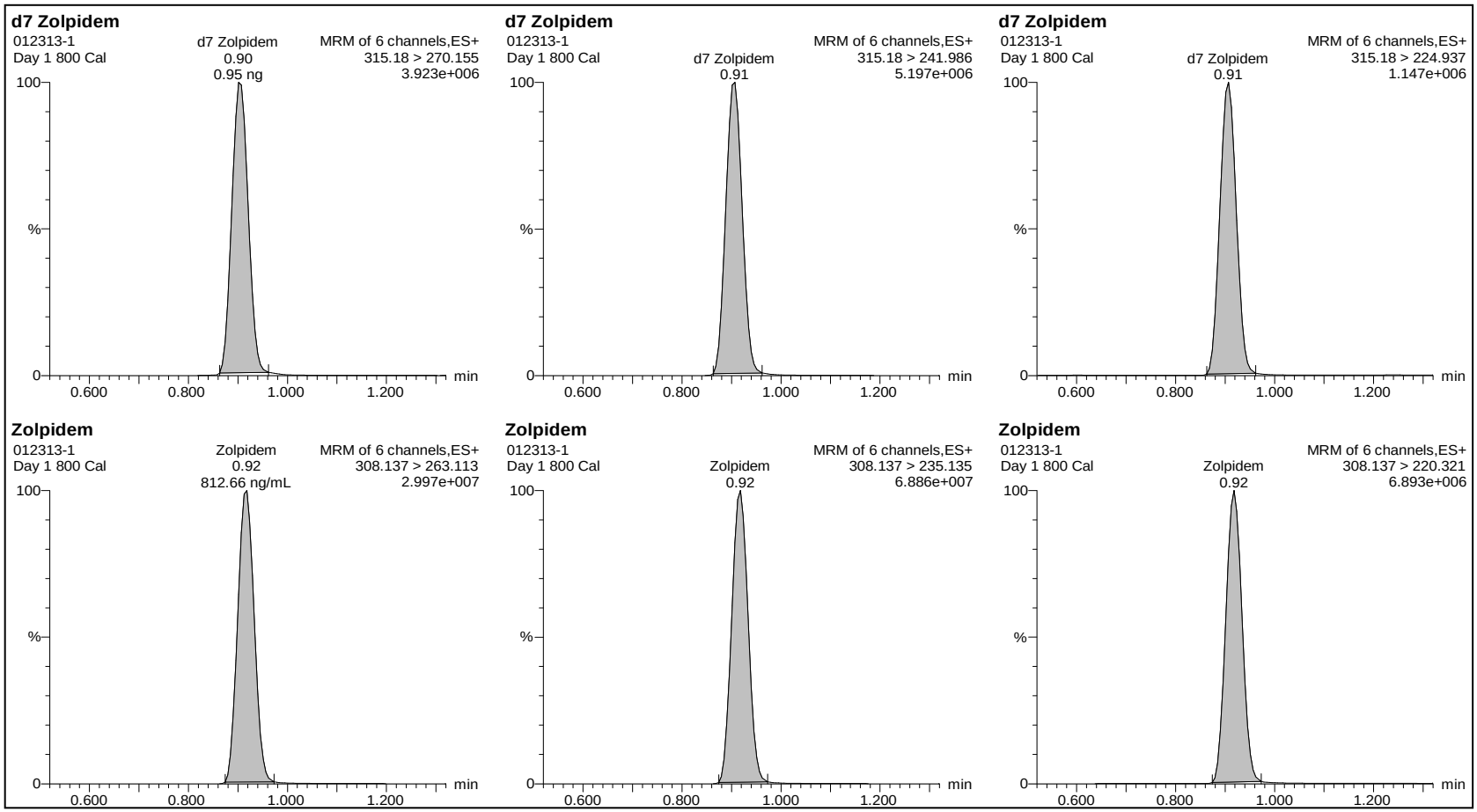

Table 3

LDR, LOD, LOQ and Recovery for Zolpidem

Compound	LDR (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Extraction Efficiency (%) ± SD*		
				1 ng/mL	100 ng/mL	500 ng/mL
Zolpidem	0.39 - 800	0.20	0.39	78 ± 2	83 ± 2	87 ± 2

* n=5 for all measurements

Blood controls were prepared at concentrations of 1, 10, 100, and 500 ng/mL and used to determine accuracy and precision, both intra-day (within day) and inter-day (between day). The control values were selected as being a representative dispersal across the LDR of zolpidem. The controls were prepared on day 1 of the experiment and were stored at 4ºC until use.

The intra-day and inter-day accuracy and precision are listed in Table 4. Accuracy, expressed as relative error (%E), was determined by calculating the difference between the target value and the measured value. Precision was expressed as the coefficient of variation (CV) of multiple analyses at a given control level.

The stability of zolpidem in bovine blood stored at 4ºC was determined by evaluating the control concentrations on day 7 and day 43 of the experiment (Table 4). Long term stability of zolpidem stored at -20ºC was evaluated by reviewing the Bioaeronautical Sciences Research Laboratory QC data. A zolpidem blood control was prepared at a target value of 0.100 µg/mL. Over a 3 year period, this QC material was analyzed 8 separate times with a mean of 0.090

μg/mL ± 0.006. This QC material was analyzed 5 times during this research project yielding a mean of 0.086 μg/mL ± 0.001.

LC-MS matrix effect, i.e., ion suppression or enhancement, was determined by analyzing multiple solvent and blood samples, and comparing their response. If the matrix control has a lower signal than the solvent control, that is ion suppression matrix effect. If it has a higher signal than it is ion enhancement matrix effect. The matrix effect is listed in Table 5.

Table 4

Intra and Inter-Day Accuracy and Precision

	Day 1			Day 2			Day 3			Day 7			Day 43		
Target (ng/mL)	Mean (ng/mL)	CV%	%E	Mean (ng/mL)	CV%	%E	Mean (ng/mL)	CV%	%E	Mean (ng/mL)	CV%	%E	Mean (ng/mL)	CV%	%E
1	1.09 ± 0.04	4	9	1.07 ± 0.04	4	7	1.07 ± 0.03	3	7	1.06 ± 0.03	3	6	1.34 ± 0.17	13	34
10	10.3 ± 0.08	1	3	10.5 ± 0.16	2	5	10.6 ± 0.20	2	6	10.6 ± 0.22	2	6	10.3 ± 0.14	1	3
100	104 ± 1.10	1	4	105 ± 1.16	1	5	106 ± 1.86	2	6	106 ± 1.36	1	6	104 ± 0.64	1	4
500	511 ± 3.70	1	2	526 ± 9.42	2	5	518 ± 6.40	1	4	515 ± 5.98	1	3	513 ± 5.21	1	3

Note. n=5 for all measurements. Accuracy measured as relative error (%E) from target concentration. Precision measured as CV in replicate measurements.

Table 5

Matrix Effect

	Matrix Control				Solvent Control		
	Compound	Int Std	Response Ratio		Compound	Int Std	Response Ratio
1	9200000	761000	12.089	1	8950000	807000	11.090
2	9090000	775000	11.729	2	8800000	765000	11.503
3	9210000	771000	11.946	3	8940000	776000	11.521
Average	9166667	769000	11.921	Average	8896667	782667	11.371
S.D.	54365	21779	0.181	S.D.	68475	17783	0.243
R.S.D.	0.593	2.832	1.521	R.S.D.	0.770	2.272	2.141
Recovery	104.8						
S.D.	2.8						
RSD	2.6						

Table 6

Postmortem Specimen Concentrations

Case	Blood	Urine	Liver	Spleen	Brain	Kidney	Muscle	Heart	VH *	Lung
1	0.077	-	0.236	0.123	0.041	0.122	0.016	0.091	0.028	0.099
2	0.028	-	0.081	0.048	0.003	0.030	0.016	0.035	-	0.040
3	0.008	-	0.014	0.016	0.003	0.014	0.003	0.003	-	0.013
4	0.015	0.014	0.040	0.014	0.008	0.012	0.005	0.007	-	0.024
5	0.052	-	0.084	0.070	0.029	0.064	0.020	0.039	-	0.069
6	0.025	-	0.032	0.021	0.007	-	0.007	0.016	-	0.028
7	0.018	0.036	0.031	0.023	0.011	0.027	0.008	0.032	-	0.029
8	0.021	-	0.035	0.032	0.013	0.028	-	0.004	-	0.021
9	0.018	0.012	0.045	0.030	0.012	0.028	0.010	0.020	-	0.033
10	0.008	-	0.027	-	-	0.017	-	-	0.002	-

Note. - Specimen type not available for analysis. All concentrations are shown in units of µg/mL or µg/g.

* VH is Vitreous Humor

Table 7

Distribution Coefficients of Zolpidem in Postmortem Specimens

Case	n=3 Urine	n=10 Liver	n=9 Spleen	n=9 Brain	n=9 Kidney	n=8 Muscle	n=9 Heart	n=2 VH*	n=9 Lung
1	-	3.084	1.609	0.537	1.600	0.214	1.188	0.370	1.292
2	-	2.886	1.708	0.121	1.082	0.580	1.238	-	1.427
3	-	1.947	2.039	0.447	1.803	0.368	0.421	-	1.697
4	0.919	2.651	0.906	0.523	0.846	0.309	0.450	-	1.584
5	-	1.625	1.344	0.555	1.242	0.377	0.754	-	1.329
6	-	1.316	0.856	0.292	-	0.284	0.624	-	1.100
7	1.978	1.705	1.273	0.574	1.448	0.443	1.749	-	1.557
8	-	1.627	1.500	0.651	1.316	-	0.175	-	0.995
9	0.701	2.565	1.689	0.650	1.554	0.576	1.113	-	1.887
10	-	3.558	-	-	2.156	-	-	0.208	-

Note. - Specimen type not available for analysis.

* VH is Vitreous Humor

The postmortem concentration of zolpidem in tissue and fluid specimens is listed in Table 6. The concentration of each specimen is listed by case and specimen type. The postmortem distribution coefficient (specimen/blood) located in Table 7, was determined from the tissue and fluid values in Table 6. The mean, standard deviation, and CV of the calculated distribution coefficients are listed in Table 8. The standard deviation shows how much variation the concentration is from the mean. The lower the standard deviation, the closer the concentration population is to the mean. The CV, coefficient of variation, is the ratio of the standard deviation to the mean.

Table 8

Mean Distribution Coefficients of Zolpidem in Postmortem Specimens

	Urine/ Blood	Liver/ Blood	Spleen/ Blood	Brain/ Blood	Kidney/ Blood	Muscle/ Blood	Heart/ Blood	VH*/ Blood	Lung/ Blood
n	3	10	9	9	9	8	9	2	9
Mean	1.2	2.3	1.4	0.5	1.4	0.39	0.86	0.29	1.4
s.d.	0.56	0.71	0.36	0.16	0.37	0.12	0.47	0.08	0.27
CV%	46	31	25	33	25	31	55	28	18

* VH is Vitreous Humor

Chapter V. Discussion

When it comes to aviation safety, a pilot's ability to safely command an aircraft is of great importance. Of equal importance is the determination of safety issues following a fatal accident. From a toxicology standpoint, the determination of the presence of a drug(s) and interpreting the effect of that drug is also of the utmost importance. Drug effects on a person are typically interpreted from the drug concentration in the blood/plasma. However, only approximately 70% of the cases received at the Bioaeronautical Sciences Research Laboratory contain blood. Therefore, it is desirable for the Laboratory to be able to make a rough estimate of a drug blood concentration from the available non-blood specimens.

Since scientific information concerning the postmortem distribution of zolpidem at therapeutic levels is not available, this research project was designed to determine its distribution in various postmortem tissues and fluids. A search of the Toxicology and Accident Research Laboratory database identified 10 fatal aviation cases that were reported positive for zolpidem in blood, with 9 of the cases having most of the full complement of biological tissues and fluids, including muscle, liver, kidney, lung, spleen, brain, heart, urine, blood, and vitreous humor, available for analysis. A method was then developed utilizing a Waters®Xevo®TQ-S Acquity UPLC®system. A simple and rapid sample preparation procedure was developed. The analytical parameters associated with the method were determined, such as the LDR, LOD, LOQ, and recovery. The relative error and CV were calculated for the intra-day and inter-day analyses to determine the accuracy and precision of the method. Finally, postmortem cases were analyzed and the distribution coefficients were evaluated for their possible suitability in estimating a blood concentration. This report details the quantitation and distribution of zolpidem in postmortem

samples and identifies specimens that may be suitable for estimating blood concentrations of zolpidem.

Method Development

Three instruments were evaluated for the analysis of zolpidem in postmortem specimens. The first instrument considered was the GC-MS. The present method for zolpidem analysis at the Bioaeronautical Sciences Research Laboratory is GC-MS, with solid phase extraction (SPE) used for sample preparation. Analyses were performed on an Agilent 7890A GC equipped with an Agilent 5975C MSD. The column was a Restek Rxi-1 ms, 12m x 0.2mm I.D., and 0.33 μm film thickness. The oven profile was 130°C – 290°C at 30°C/min and the injector temperature was 250°C. The sample injection was 1 μL in the splitless mode. The retention time was 5.57 min for zolpidem and 4.24 min for the IS (SERTIS), with a total run time of 6.33 min. The fragmentation of the deuterated zolpidem drug standard yielded similar ions to that of the non-deuterated zolpidem standard, therefore SERTIS was used for the IS. The use of a non-structurally related IS is not suitable for the analysis of various matrices against a blood curve, due to the differing extraction efficiencies of the 2 drugs in different matrices. This fact makes any quantitative information based on the analysis of zolpidem in non-calibrant matrix highly suspect.

LC-MS was another instrument considered. The instrument used for the analysis was a Thermo LTQ XL LC with an Agilent 1200 Series MS. The column was an Ascentis Express C18, 10cm x 4.6mm, 2.7μm. At a flow rate of 0.800 mL/min the retention time was 1.04 min for both zolpidem and sertis (IS) with a total run time of 4 min. An SPE, as used in the GC-MS

method above, provided reasonable sensitivity towards the analytes of interest. However, SPE is time-consuming, laborious and costly. A simplified sample preparation using a filter vial was attempted. A filter vial is a 2 piece LC vial with a filter incorporated into the top "plunger" part of the vial. The filter physically separated the sample's proteins and debris from the sample's liquid using a 0.20 μm filter. The actual sample preparation was a crash and shoot methodology involving 200 μL of sample and 200 μL of acetonitrile, followed by filtration. The filter vial worked well on the LC-MS for liquid samples; however, the tissues were unsuccessful.

The third instrument considered, and the one selected, was the UPLC-MS. The instrument was a Waters®Xevo®TQ-S Acquity UPLC®system. The column was an Acquity UPLC® BEH C18 1.7 μm, 2.1 x 100mm. The small column particle size associated with UPLC generates narrow peaks and enhanced chromatographic resolution reducing the chances of co-eluting interferences (Nielsen & Johansen, 2012) (Humbert, Grisel, Richeval, & Lhermitte, 2010). The sample injection volume was 2 μL. At a flow rate of 0.40 mL/min, the retention time for zolpidem was 0.92 min and 0.91 min for zolpidem d7. Zolpidem d7 was the IS for the method. Following use, the column was washed and stored in a 20:80 mixture of methanol:H_2O. The same SPE for the GC-MS method was attempted and was successful. However, as discussed earlier, SPE is costly and time consuming. Therefore, we evaluated various crash and shoot sample preparations.

The filter vial, since unsuccessful on the LC-MS, was not attempted on the UPLC-MS. A simplified version of the crash and shoot method was evaluated. Variables to be investigated in a crash and shoot sample preparation include the concentration of sample and IS to be used. All samples were crashed with 1 mL acetonitrile, 0.20 mL of supernatant was transferred, dried down and brought up in 1 mL of 70:30 (B:A), which matched the UPLC mobile phase. Starting

with 0.5 mL of the sample and 100 ng IS, the instrument and column were both overloaded. After reducing the amount of sample to 0.10 mL, control samples at therapeutic zolpidem concentrations were able to be seen without overloading the instrument or the column. The initial amount of IS was 100 ng, which overloaded the instrument and column. The IS was then reduced to 25 ng, but overloading of the instrument and column still occurred. Finally, 10 ng was determined to be an appropriate amount, both for the MS and the column. Additionally, 10 ng of IS represented the total sample concentration of zolpidem expected from a sample at a mid-point in the calibration curve, i.e., 0.10 mL of a 100 ng/mL sample is a total of 10 ng zolpidem. In summary, a simple and rapid crash and shoot (protein precipitation) method was employed using 0.100 mL sample, 0.100 mL IS (10 ng), and 1 mL cold acetonitrile, centrifuged at 1500xg, 0.200 mL isolated, dried and reconstituted in 1 mL of 70:30 (water:acetonitrile). This crash and shoot method provided excellent recovery and sensitivity (discussed below). The crash and shoot (protein precipitation) extraction on the UPLC-MS was determined to be the ultimate way to perform this experiment as it was less labor intensive and costly with rapid, accurate, and precise results.

The Method Development Report generated by the Waters Intellistart™ Technology, optimized the mass spectrometer portion of the LC-MS. Intellistart evaluated the 4 modes of compound ionization (APCI ± and ESI ±) and determined that ESI+ provided the maximum ionization for the analytes of interest. The optimal cone voltage and collision energy for each were also specified. Four product ions were provided for each of the desired analytes, zolpidem and zolpidem d7. Zolpidem has a molecular weight (MW) of 307.4. The precursor ion (M+H) was m/z 308.1. The product ions listed by Intellistart were m/z 235.1, 220.1, 248.1, and 205.2. Ion m/z 263.1 was previously used in the Bioaeronautical Sciences Research Laboratory as a

zolpidem product ion, even though the Water's Intellistart did not identify it as such. For zolpidem d7, with a MW of 314.4, the precursor ion was m/z 315.2. The product ions listed by Intellistart were m/z 242.0, 270.2, 224.9, and 252.1. With this information, samples were run to determine which of the product ions to use. A review of each ion chromatogram enabled the selection of the most suitable ions. The ions were selected based on abundance and mass-to-charge ratio (m/z). High mass ions were preferable in order to reduce interference and increase reproducibility.

Actual samples were analyzed collecting all of the proposed ions. The 2 product ions selected for zolpidem were m/z 263.1 and 235.3. Ion 263.1 was the quant ion and 235.3 was the qualifier ion for this method. The other product ions suggested by Intellistart were not as consistent as ions 263.1 and 235.3. All of the product ions for zolpidem d7 were evaluated and the ions selected for use were m/z 270.2 as the quant ion and m/z 242.0 and 224.9 as qualifier ions (Tables 1 and 2). The optimal cone voltage for all zolpidem product ions was 2 and the collision energies varied. The cone voltage for zolpidem d7 product ions was 42, also with varying collision energies. The final MS parameters were: cone voltage of 42; collision energies of 38.0 for m/z 220.1, 36.0 for m/z 235.1, 26.0 for m/z 263.1, 38.0 for m/z 224.9 and m/z 242.0, and 26.0 for m/z 270.2.

The limit of detection (LOD), limit of quantitation (LOQ) and linear dynamic range (LDR) for each analyte are listed in Table 3. The LOD was defined as the lowest concentration of analyte having a minimum signal-to-noise (S/N) ratio of 5, having a product ion ratio ± 20% of the calibrator average, and ± 2% retention time of the calibrator average. The LOQ was defined as meeting all LOD criteria plus having an S/N ratio of 10 and having a measured value,

compared to calibrators, within ± 20% of its target concentration. A calibration curve was prepared in bovine whole blood at concentrations from 0.02 – 800 ng/mL. The LOD was found to be 0.20 ng/mL and the LOQ was found to be 0.39 ng/mL. The LDR for zolpidem was 0.39 - 800 ng/mL. The correlation coefficient of the LDR curve exceeded 0.9999

Carryover from one sample to the next was not found to be a problem. It was, however, initially investigated and subsequently monitored by the use of solvent injections. Acetonitrile blanks were injected following the 800 ng/mL calibrator, as well as after each set of controls. There was no sample-to-sample contamination. Subsequently, blanks were used randomly throughout the sample sequence to verify that no sample-to-sample contamination occurred.

The extraction efficiency of zolpidem at various concentrations obtained from this crash and shoot (protein precipitation) method was high, ranging from 77% - 87%. The extraction efficiency was 77.5 ± 1.8 % at 1 ng/mL, 83.1 ± 2.1 % at 100 ng/mL, and 87.3 ± 2.0 % at 500 ng/mL (Table 3). These values are exceptional considering the simplicity of the extraction procedure, and in comparison to the extraction efficiency found in other studies (Johnson, R. D. and Lewis, R. J., 2005) (Johnson, R. D. and Lewis. R. J., 2009) (Lewis, R. J., Johnson, R. D., Angier, M. K., Ritter, R. M., Drilling, H. S., and Williams, S. D., 2003) (Reddy, D. C., Bapuji, A. T., Suryanarayana Rao, V., Himabindu, V., Raju, D. R., Syedba, S., Ravinder, S., Ravikiran, H. L. V., 2011).

The accuracy and precision of the method were evaluated by an intra-day and inter-day study. The study was performed using bovine blood controls at concentrations of 1, 10, 100 and 500 ng/mL. They were prepared on day 1 of the experiment and stored at 4°C until extracted. All days were compared to the day 1 calibration curve. The accuracy of the method was

evaluated by determining the measured control values compared to the target value (expressed as % relative error). The precision, or reproducibility of a measurement, was measured by the CV (relative standard deviation) of multiple analyses of the same control.

The intra-day relative error in the 1, 10, 100 and 500 ng/mL control range were ≤ 9%. The intra-day CV values were ≤ 4%. The inter-day relative errors for days 2 and 3 were ≤ 7%, and the CV values were ≤ 4%. The intra-day and inter-day results indicate this method is both accurate and precise over a 3 day period (Table 4).

The short term stability of zolpidem in bovine blood stored at 4°C was determined by evaluating the control concentrations obtained on day 7 (Table 4). The inter-day relative error for 1, 10, 100 and 500 ng/mL controls at day 7 were ≤ 6%. The CVs for day 7 were ≤ 3%. These values were determined from the day one calibration curve. There was no apparent decrease in concentration after 1 week of storage at 4°C at any of the control concentrations tested. This study demonstrates that bovine blood specimen's positive for zolpidem should be stable for up to 7 days when stored at 4°C. However, to ensure the highest quality analytical data, and as good laboratory practice, it is recommended that biological specimens be analyzed promptly after thawing.

For long term stability of zolpidem in bovine blood stored at 4°C, another analysis was performed on day 43 (Table 4). At day 43, the relative error for the 1 ng/mL control was 34%, but the 10, 100 and 500 ng/mL controls were ≤ 4%. The CV's for the 1 ng/mL on day 43 was 13%, and the CV's for the 10, 100 and 500 ng/mL were ≤ 1%. Based on the 10, 100 and 500 ng/mL there was no apparent decrease in concentration after 43 days (about 6 weeks) of storage at 4°C. The 1 ng/mL control was out of the acceptable range on the high end, but at that low

concentration it does not require much of an operator error to display this size of error. Any number of things, such as a pipetting error or air bubble, could cause this difference. The day 43 values were determined from the day one calibration curve, which could easily be a factor as determining quantitative values from a 43 day old curve is not typically acceptable. The study demonstrated that the long term stability of zolpidem spiked bovine blood should be stable for up to 6 weeks when stored at 4°C; however, this is not recommended.

For long term stability of zolpidem in bovine blood stored at -20°C, the concentration history of a Bioaeronautical Sciences Research Laboratory zolpidem QC control, with a target concentration of 0.100 μg/mL, was evaluated. This quality control was prepared and analyzed 8 separate times over a 3 year period, with a mean of 0.090 μg/mL ± 0.006. Furthermore, this blind control was obtained from the QC department and analyzed 5 separate times with mean results of 0.086 μg/mL ± 0.001. These results confirm the long term stability of zolpidem in bovine blood stored at -20°C.

Matrix effect is of great concern in LC-MS. Co-eluting matrix can cause ion suppression or ion enhancement. In this study the matrix effect was determined by preparing 2 concentration-identical controls – 1 in solvent (acetonitrile) and 1 on a sample matrix (blood in this study). The ratio between the analyte area and IS area of the matrix (11.92 ± 0.18) and the solvent samples (11.4 ± 0.24) gave the matrix effect. The matrix and solvent samples were analyzed 3 separate times. The determined matrix to solvent ratio was 105 ± 3. This result indicates that there is essentially no statistical matrix effect (Table 5).

Postmortem Sample Analysis

Specimens from fatal aviation accident victims are routinely sent to the Bioaeronautical Sciences Research Laboratory for toxicological analysis. Postmortem tissues and fluids obtained from 10 separate aviation fatalities (years: 2007-2010) that had previously screened positive for zolpidem were re-examined using the current method. The fluid and tissue specimens examined from each victim, if available, included: blood, urine, liver, spleen, brain, kidney, muscle, heart, vitreous humor, and lung.

The pharmacology, pharmacokinetics, and pharmacodynamics of zolpidem are beyond the scope of this thesis. However, extensive discussions can be found elsewhere (Lewis, Angier, Johnson, Rains, and Nepal, 2011). Therapeutic blood concentrations for zolpidem range from 0.080 to 0.150 µg/mL (Uges, 2004). Toxic levels of zolpidem have been reported at approximately 0.500 µg/mL (Uges, 2004). Lethal levels of zolpidem concentrations begin in the 2.00 to 4.00 µg/mL range (Uges, 2004). Blood concentrations observed in the current study ranged from 0.008 – 0.077 µg/mL, which appear to be in the sub-therapeutic to low therapeutic concentration range. With the short half-life of zolpidem, low concentrations would be expected in toxicological samples of postmortem specimens from aviation accidents if the zolpidem ingestion were from the "night before". Since the site from which the blood was collected at autopsy is unknown for each of these cases, and due to postmortem redistribution or other factors, these blood concentrations may not be representative of the levels observed prior to death.

The concentration of zolpidem in each postmortem specimen analyzed from these 10 cases, are presented in Table 6. The following mean concentration (µg/mL, µg/g) of zolpidem was detected in each specimen type: blood 0.027 (range 0.008 – 0.077, n=10), urine 0.021 (range

0.012 – 0.036, n=3), liver 0.063 (range 0.015 – 0.236, n=10), spleen 0.042 (range 0.014 – 0.123, n=9), brain 0.014 (range 0.003 – 0.041, n=9), kidney 0.038 (range 0.013 – 0.122, n=9), muscle 0.011 (range 0.003 – 0.020, n=8), heart 0.027 (range 0.003 – 0.091, n=9), vitreous humor 0.015 (range 0.002 – 0.028, n=2), and lung 0.039 (range 0.013 – 0.099, n=9).

On average, the highest concentrations of zolpidem present in each victim were found in liver, spleen, kidney, and lung tissue specimens. The general trend for highest concentration to lowest concentration of zolpidem was: liver, spleen, lung, kidney, blood, heart, urine, vitreous humor, brain, and muscle. The high tissue concentrations were surprising since the volumes of distribution (V_d) for zolpidem is so small, ranging from 0.54 to 0.68 L/kg.

The distribution coefficients for zolpidem, expressed as specimen concentration/ blood concentration, are listed in Table 7. The summary of distribution coefficients located in Table 8 are: urine 1.2 ± 0.56, liver 2.3 ± 0.71, spleen 1.4 ± 0.36, brain 0.50 ± 0.16, kidney 1.4 ± 0.37, muscle 0.39 ± 0.12, heart 0.86 ± 0.47, vitreous humor 0.29 ± 0.08, lung 1.4 ± 0.27. The %CV values ranged from 18 – 55%. Basic drugs with large V_d can undergo postmortem redistribution. However, zolpidem has a very low V_d, which may partially explain the relatively low CVs observed for some of the postmortem distribution coefficients. Given the relatively low CV's of the distribution coefficients for some of the tissues, postmortem redistribution may not have been significant in all tissues.

The large CV's associated with the distribution coefficients for most tissues and fluids were not completely unexpected, as many unknown variables exist in these cases. These variables include such things as differing blood collection sites at autopsy, postmortem interval, postmortem redistribution, contamination, time from drug administration to death, and dosage,

just to name a few. The blood typically is noted as having been collected from the chest cavity in most of the cases we receive for analysis when the collection site is reported.

Drug concentrations in blood may aid in determining drug impairment. However, the Bioaeronautical Sciences Research Laboratory receives blood in only approximately 70% of cases. There are no widely accepted criteria for what constitutes an acceptable distribution coefficient; however, it may be possible, with extreme caution, to use a tissue or fluid distribution coefficient to crudely estimate a blood concentration in cases where blood is not available, if the distribution coefficient has a CV of $\leq 25\%$. Therefore, the results obtained from our limited number of cases suggest that zolpidem concentrations found in lung, kidney, and spleen could be used with caution to estimate blood zolpidem concentrations ranging from sub-therapeutic to therapeutic levels. While a study involving a greater number of samples from a larger pool of cases needs to be completed to more definitively verify these results, based on these findings one could cautiously estimate a range for postmortem zolpidem concentrations in blood.

Conclusions

In conclusion, it was first confirmed that zolpidem was indeed a drug that should be researched for postmortem distribution. The impairments caused by the drug are numerous to include, but not limited to, drowsiness, dizziness, amnesia, memory impairment, nausea, slow and slurred speech, diminished reflexes, and lack of coordination, nightmares, hallucinations, leg cramps, double vision, and dry mouth. With a therapeutic dose, these effects can last up to 4-5 hr. If used concurrently with another CNS depressant drug, the effects may last as long as 8-16

hr (Couper & Logan, 2010). Ingesting a therapeutic dose of zolpidem at bedtime may or may not present an issue the next day. However, there are numerous cases with accidental ingestion during the day, as well as being used for a drug facilitated sexual assault (DFSA), drug facilitated assault (DFA), or causing DUID cases. It was also confirmed there had not been a study performed with the expanded list of specimen types, or with the lower therapeutic values as were anticipated and found in this study.

In cases where blood is not available, such as approximately 30% of the FAA's Bioaeronautical Sciences Research Laboratory cases, it has been suggested by Lewis, et.al. (2007) that it may be possible to use either tissue or fluid concentrations to estimate, with caution, a blood concentration. This may be possible if the tissue or fluid distribution coefficient(s) have a CV of $\leq 25\%$. Taking this into consideration, the study results suggest the concentrations of zolpidem found in the spleen, kidney, and lung could be used, with caution, to estimate blood zolpidem concentrations. The CV of these tissue specimens were 25%, 24% and 18%, respectively.

There were 2 research questions for consideration in this study. The first, was there a consistent postmortem distribution of zolpidem in postmortem blood and unique body tissues and/ or other body fluids? Based on the results of this study, yes, there is a consistent postmortem distribution of zolpidem found in the spleen, kidney, and lung within this study. The second question, would it then be possible to estimate zolpidem blood concentrations from the zolpidem fluid and tissue concentrations? Yes, it would be possible to roughly estimate, with caution, zolpidem blood concentrations from the zolpidem spleen, kidney, and lung concentrations.

Limitations and Recommendations for Future Research

The limitations of this study were the small sample size and the lack of autopsy information. Numerous positive zolpidem cases were identified in the CAMI database; however, only 10 cases contained the specimen types necessary for this study. The precise blood collection site(s) and postmortem interval for the cases are unknown. However, in most of the cases received at the Bioaeronautical Sciences Research Laboratory the chest cavity is noted as having been the collection site. The sample size limitation can be lessened by performing further studies over time. It is doubtful, however, that the autopsy information limitation could be overcome as the specimens are received from numerous locations.

Future research could be an extension of this project, looking at additional cases, perhaps, every 3 or 4 years, as more cases are available with the desired tissues and fluids. The addition of cases to the study would be beneficial in resolving the small sample size limitation of the study.

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