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Validation and Application of a Liquid Chromatography-Tandem Mass Spectrometry Method for Mitragynine in Postmortem Blood and Liver

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Validation and Application of a Liquid Chromatography-Tandem Mass Spectrometry Method for Mitragynine in Postmortem Blood and Liver

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Abstract

Kratom is a tree originating in Southeast Asia, and has a history of usage among farmers. The leaves of the tree were chewed and used by farmers as a stimulant in order to work long days. Over the years, the popularity of the drug spread worldwide and has risen in recent years. In 2016, kratom was put on a Drugs of Concern list by the United States Drug Enforcement Administration (DEA). A lack of research including studies for mitragynine and 7-hydroxymitragynine has been observed, and there are even fewer postmortem case studies in the literature. The rise in kratom usage was noticed in the casework of the Office of the Chief Medical Examiner (OCME) Toxicology Laboratory in Oklahoma.

The number of cases involving mitragynine, an alkaloid in kratom, has risen over recent years, but the OCME did not have a validated method for quantitation. The importance of this thesis project was to validate a method for the quantitation of mitragynine and qualitative identification of 7-hydroxymitragynine, assess the stability of the two alkaloids in three different storage conditions (ambient, refrigerated, and frozen), and examine real world cases coming through the OCME. The extraction utilized in this method was a phosphate buffer followed by filtering the samples using 3 mL columns containing diatomaceous earth. Extracts were then concentrated by drying them down, reconstituted, and washed with hexanes. The samples were then analyzed using liquid chromatography paired with tandem mass spectrometry (LC/MS-MS).

The method for quantitation of mitragynine was validated with the criteria of interferences, bias, precision, ion suppression/enhancement, limit of detection, carryover, calibration model, and stability taken into consideration. The validation criteria for 7-hydroxymitragynine were interferences, ion suppression/enhancement, limit of detection, carryover, and stability. All criteria were met and deemed acceptable by the OCME lab's Quality Assurance Program.

The stability study demonstrated that mitragynine is more stable in refrigerator conditions as opposed to freezer, and that 7-hydroxymitragynine went through an approximate 20% decrease over 65 days in the refrigerator in blood. The case studies had ranges of 130-1,400 ng/mL for heart blood, 130-1,300 ng/mL for femoral blood, and 220-7,000 ng/mL for liver. Central/Peripheral (C/P) and Liver/Peripheral (L/P) ratios were calculated to identify the potential for mitragynine to undergo postmortem redistribution (PMR). The C/P and L/P ratios were an average of 1.0 and 2.7 respectively, suggesting that mitragynine does not experience significant PMR. This thesis project will be adding to the body of knowledge concerning mitragynine and 7-hydroxymitragynine which will help researchers and postmortem labs assess the storage conditions of their specimen and help with interpretation of toxicology results in death investigations.

Introduction

Kratom is a common name used interchangeably for the tropical tree, also known as Korth, in addition to the compounds derived from the plant. Korth is indigenous to Thailand, Malaysia, Myanmar, and Southeast Asia and shown in Figure 1. Extracts from the tree contain 44 alkaloids including mitragynine, paynantheine, speciogynine, speciociliatine and 7-hydroxymitragynine (7-OH) which are the most abundant in the plant. Mitragynine and 7-OH have been researched more than the other alkaloids as mitragynine is the most abundant active alkaloid in kratom while 7-hydroxymitragynine has a greater affinity for opioid receptors. (S. Basiliere & Kerrigan, 2020a; Trakulsrichai et al., 2015).

Figure 1. Photo of kratom tree (DEA, 2020).

In 2016, the DEA put the drug on a list of "Drugs and Chemicals of Concern" and intended to assign it as a Schedule I drug. The intention was declared by the DEA because of an increase in law enforcement seizures of kratom, the high potential for abuse, and the fact that no accepted medical use was found in the United States. The intent was withdrawn later that year due to public demand that the drug stay legal (Singh, Narayanan, & Vicknasingam, 2016). In 2018, it was found that over 2 million adults in the United States of America (U.S.) used kratom in a 12-month span (Schimmel et al., 2021). The usage of kratom in the U.S. is rising; therefore, the reported medical outcomes and health effects could lead to an emerging public health threat (Anwar, Law, & Schier, 2016).

Statement of Problem

According to the National Institute on Drug Abuse (NIDA), there has not been uses for kratom approved by the U.S. Food and Drug Administration (NIDA, 2023). Even without this approval, the NIDA reported that people used kratom to manage drug withdrawal symptoms and

cravings, combat pain, fatigue, and mental health problems. (National Institute on Drug Abuse, 2023) The Office of the Oklahoma Chief Medical Examiner (OCME) Toxicology Laboratory has seen an increase in mitragynine detections over the last four years. The prevalence of mitragynine in OCME cases has represented a potential public health threat due to its recent rise in use. The lab previously did not have the means to develop and validate an in-house method and relied upon third-party outsource labs to provide testing for mitragynine.

The new and validated in-house method was developed to save the OCME thousands of dollars annually by not having to outsource a mitragynine quantitation and as well decreased turnaround time for any death investigation cases involving mitragynine. Outsourcing increased the case's turnaround time which resulted in decedent's families waiting longer to receive results and longer delays for pathologists to rule on the cause and manner of death. The new method was developed to further facilitate the OCME to meet their mission by assisting families, law enforcement, and Oklahoma taxpayers in a timely and frugal manner. The OCME Toxicology Laboratory is accredited by the American Board of Forensic Toxicology (ABFT) and uses methodologies drawn from the Academy Standards Board (ASB) recommendations.

Purpose of Study

Limited research is available regarding postmortem concentrations of mitragynine and identification of 7-OH in forensic cases. Therefore, the new validated method will be performed on routine casework received by the OCME and the data obtained will add to the growing body of knowledge regarding kratom. By analyzing heart and femoral blood and liver, the laboratory evaluated PMR, a common phenomenon known within the field of postmortem toxicology. PMR has been described as a change in drug concentration that occurs after death. It is the postmortem movement of drugs from areas of high concentration (e.g. liver, lungs, heart) to areas of low

concentration (e.g. blood). The data obtained from the method application will further aid toxicologists in interpreting medicolegal death investigation cases positive for mitragynine.

Scope of Study

In this thesis project, a LC/MS-MS method to quantitate mitragynine and qualitatively identify 7-OH in blood and liver was validated. A stability study was also completed to assess the stability of mitragynine and 7-OH in blood under three different storage conditions, including room temperature, refrigerated, and frozen. Within the validation methodology the processed stability of the analytes in the autosampler conditions over 24, 48, and 72 hours was also assessed.

The validated method was then applied to authentic forensic casework. The cases which were analyzed for mitragynine, and 7-OH were identified from regular OCME casework within the timeframe of the thesis project. The forensic cases were tested for mitragynine and 7-OH in heart blood, femoral blood, and liver samples, when available, to assess the postmortem distribution and redistribution of the analytes in real world scenarios.

Significance to the Field

Mitragynine deaths have increased over the last few decades and knowledge about stability and postmortem concentrations is scarce. Published data regarding the stability of mitragynine and 7-OH in blood is limited at this time (S. Basiliere & Kerrigan, 2020b; Kerrigan & Basiliere, 2022; Manda et al., 2014; Papsun et al., 2019; Parthasarathy et al., 2010; Ya, Tangamornsuksan, Scholfield, Methaneethorn, & Lohitnavy, 2019). Additionally, there is little published data concerning real world postmortem cases for mitragynine (Domingo et al., 2017; Karinen, Fosen, Rogde, & Vindenes, 2014; Mata & Andera, 2020; I. M. McIntyre, Trochta, Stolberg, &

Campman, 2015). The data from this thesis project will add to the body of knowledge concerning mitragynine and 7-OH and assist in the assessing of casework.

The knowledge of drug stability in blood will help future forensic chemists and toxicologists determine the urgency in testing a case for mitragynine within a certain time frame. If the drug in the sample has degraded before quantitation has been completed, then interpretations of that concentration will not be accurate or reliable. In forensic toxicology, the accurate concentration of a drug is essential to determining the probable cause of death in such cases. Since kratom is legal in the United States and several other countries, the consumption of this product will likely only increase as it becomes more accessible and well-known. This thesis project establishes a validated method for blood and liver testing, stability of mitragynine and 7-hydroxymitragynine in blood, and ten case studies which will add to the available literature.

Literature Review

Mitragyna speciosa

Kratom is a drug that is found within the leaves of the tropical tree, Korth, which is indigenous to Thailand, Malaysia, Myanmar, and Southeast Asia (Cinosi et al., 2015). Common names given to the kratom plant include biak-biak and ketum in Malaysia (N A Hanapi, Ismail, & Mansor, 2013; Trakulsrichai et al., 2015). *Mitragyna speciosa (M. speciosa),* which is the scientific name for the Korth tree, is in the same family as the coffee plant (*Rubiaceae*) (Trakulsrichai et al., 2015). The leaves of the kratom tree have historically been chewed or brewed into a tea by natives of Thailand for socio-religious reasons, opiate substitution and by farmers to reduce fatigue (DEA, 2019; Graves et al., 2021). The leaves are also used in folk remedies for coughing, diarrhea, diabetes, muscle relaxation, muscle ache and hypertension (Chittrakarn, Penjamras, & Keawpradub, 2012; Ya et al., 2019).

Mitragyna speciosa is found in two different varieties based on the location of the plant, and whether red and green veins are present (N A Hanapi et al., 2013). Kratom contains 44 alkaloids including mitragynine, paynantheine, speciogynine, speciociliatine, and 7-OH which are the most abundant in the plant. Mitragynine is the most abundant alkaloid in the plant, making up 66% of the crude weight depending on the region in which it is grown (S. Basiliere & Kerrigan, 2020a; Trakulsrichai et al., 2015). Speciogynine, speciociliatine, paynantheine, and 7-OH were reported to account for 6.6%, 8.6%, 0.8%, and 2.0% respectively (S. Basiliere, 2020).

Mitragynine, also known as 9-methoxycarynantheidine, is also responsible for many of kratom's pharmacological effects (Parthasarathy et al., 2010). Speciogynine, speciociliatine, and paynantheine have not been thought to be significantly psychoactive, but not much research has been done on these alkaloids to date (S. Basiliere, 2020). Mitragynine and 7-OH exhibit agonist activity at human μ-opioid receptors. On the other hand, speciogynine, speciociliatine, and paynantheine have shown no measurable agonist activity at any of the human opioid receptors at concentrations up to 100 μM, and only weak antagonist activity was observed (Henningfield, Fant, & Wang, 2018). The primary analytes for forensic testing have been mitragynine and 7-OH since mitragynine is the most abundant active alkaloid and 7-OH exhibits more affinity for the μ-opioid receptor (S. Basiliere & Kerrigan, 2020a).

The structure of mitragynine (Figure 2) is similar to another herbal drug, yohimbine (Lydecker et al., 2016). Mitragynine is characterized as an indole because of its indole aromatic ring which is attached to two piperidine rings. 7-hydroxymitragynine (Figure 2), on the other hand, has a hydroxyl group at C-7 which increases its potency, making it 13 to 46 fold more potent than morphine and mitragynine respectively at the μ-opioid receptor (Cinosi et al., 2015). Mitragynine is a weak base (pKa, 8.1) and is lipophilic. Its lipophilicity causes the drug to have

poor solubility in water and basic media and it is acid labile (Ramanathan et al., 2015; Ya et al., 2019).

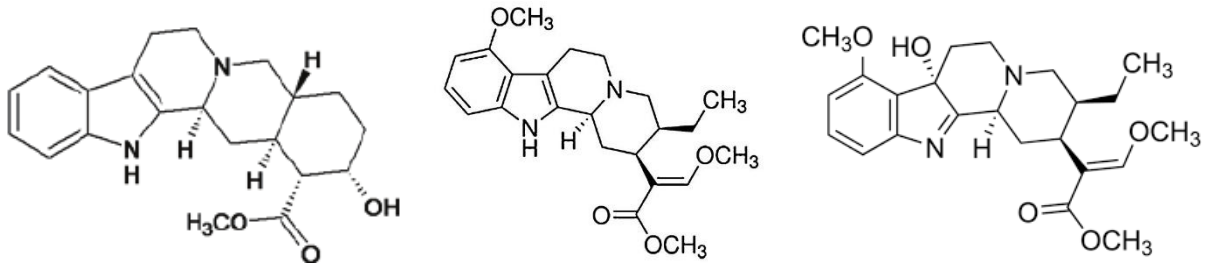


Figure 2. Structure of yohimbine, mitragynine and 7-hydroxymitragynine.

The alkaloids within the kratom tree have been reported to have stimulant effects at low doses and opiate-like effects at higher doses (Singh et al., 2016). The dose-dependent effects of the herbal drug led to the plant being used as an opium substitute in Thailand (Jansen & Prast, 1988). Even though kratom has historically been used as an opium alternative, the usage of the drug as a substitute is still controversial since research that both supports and denies the claim has been published. A study in Malaysia also reported that 55% of people that use kratom regularly develop severe dependence (Singh et al., 2016).

History

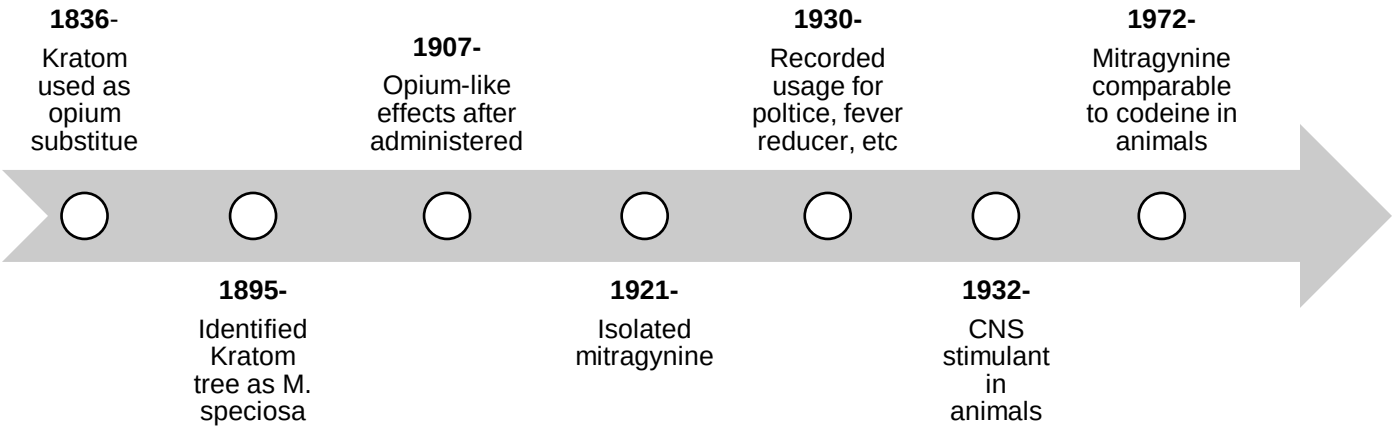


Figure 3. Timeline of important dates in the research of the alkaloid mitragynine

Jansen and Prast from the University of Auckland Medical School published a paper in 1988, which was a comprehensive recollection of the history of mitragynine research up to that time. Figure 3 depicts the main events in the history of mitragynine research summarized by Jansen and Prast. The first recorded usage of kratom extends back into the 19th century when it was used as an opium substitute by natives of Malayans in 1836, according to Jansen and Prast. Later in 1895, the kratom tree was identified as *M. speciosa* and its usage as an alternative to opium was again recorded. Different methods of administration for kratom (smoked, chewed, or drunk) were described in 1907, and noted its opiate-like effects. Two new alkaloids from the plant were then isolated in 1921, after samples of different leaves were sent to the University of Edinburgh. Mitragynine and mitraversine were both isolated from different trees (*M. speciosa* and *M. parvifolia*) (Jansen & Prast, 1988).

Jansen and Prast then reference that in 1930, different usages for kratom were recorded to be a fever reducer, "wound poultice" and suppressed "opiate withdrawal syndrome". Pharmacological research was completed on kratom in 193l. Mitragynine was found to be a central nervous system stimulant contrary to the past claims that the drug has opiate-like effects. This discovery was made by testing mitragynine on animals and reported that the drug resembled cocaine in its effects. Natives of Thailand would use mitragynine to combat fatigue from physical labor and it was noted that many addicts were thin with unhealthy complexions, dark lips, and dry skin. Mitragynine was administered to five different men and reported cocaine-like effects and stated that extracting the drug from the leaves was an efficient way to use the drug. It was found that fifty milligrams of the pure mitragynine acetate extract would produce nausea and vomiting in some subjects.

Jansen and Prast continued to describe the history of kratom in the 1970's and the structure of kratom alkaloids was elucidated and were described as "indoles and oxindoles with a closed or open E ring with substitution at the C9 position". Twenty-two alkaloids were identified in the 1970's and mitragynine's structure was compared to the indole psychedelics (psilocybin and lysergic acid amide). In 1972 mitragynine was found to be comparable with codeine as an analgesic in dogs and it did not cause emesis or dyspnea (hyperventilation) unlike codeine (Jansen & Prast, 1988).

Methods of Abuse

Historically, the leaves of the kratom tree have been chewed by locals or brewed into teas. In modern times, kratom can be prepared in many different ways including capsules, tablets, gums, and the conventional method of brewing the leaves into a tea (Singh et al., 2016). The effects of kratom are dose-dependent and it is reported that a typical user takes between 2-10 grams of plant material to achieve the desired effects. Regular users of kratom reported that their drug usage was attributed to the ability of the drug to "decrease pain, increase energy, and decrease their depressive mood" (Grundmann, 2017).

New mixtures incorporating kratom with other drugs have been marketed online or mixed by the consumer. "4X100" is a broad term for multiple products containing a blend of kratom, cola, cough syrup or diphenhydramine syrup, but the overall composition varies by the seller. This kratom cocktail has been especially popular in Thailand and is primarily consumed by teenagers and young adults. Users of the cocktail are usually looked down upon by community members and are compared to "methamphetamine and heroin addicts" (Warner, Kaufman, & Grundmann, 2016). It was found that the new preparation of kratom was used by high school students at the

same rate as cannabis. Newer versions of the "4X100" mixture also include alprazolam and coffee and are named "5X100" or "6X100" (Chittrakarn et al., 2012; Warner et al., 2016; Ya et al., 2019).

Another product containing kratom which is being sold online, is called "Krypton" and is a mixture of kratom leaves with O-desmethyltramadol, an active metabolite of tramadol. In 2016, there were 9 cases of death in less than a year which could be linked to overdosing on "Krypton" in Sweden. It has become apparent that kratom toxicity is especially present when the drug is mixed with other substances (Ya et al., 2019).

Legality/Prevalence in U.S.

The legal status of kratom in the U.S. has continued being a topic of discussion over the last decade. Even though the drug has not been scheduled it has been categorized as a "drug of concern" by the DEA (Schimmel et al., 2021). The drug has been marketed as a dietary supplement in the U.S. for over ten years, however, the Centers for Disease Control and Prevention (CDC) has reported that kratom-related calls to poison control centers increased between 2011-2015 (Grundmann, 2017). The Food and Drug Administration (FDA) still categorizes kratom as "under active review" because there is potential for abuse and harm while using the drug (Schimmel et al., 2021).

Kratom became illegal in Thailand with the passing of the Kratom Act in 1943 which made the planting of new trees illegal. In 1979, the Narcotics Act B.E. 2522 made kratom a Category V drug in Thailand. According to the New York Post, in 2021, Thailand decriminalized kratom which led to the release of 121 inmates. The drug is now legal to sell and possess, and was mainly legalized to act as a substitute for costly opiates (Associated Press, 2021). It is currently

illegal in Malaysia, Myanmar, Australia, Denmark, Romania, Sweden, Poland, Latvia, Lithuania, and New Zealand (S. Basiliere & Kerrigan, 2020a). The drug is currently under scrutiny in countries such as the United Kingdom, Germany, and the U.S. Even though the drug is under surveillance, the usage of kratom is not monitored by most national drug abuse surveys; therefore, the overall usage of kratom could be underestimated (Lu, Tran, Nelsen, & Aldous, 2009).

M. speciosa is a legal psychoactive substance in the U.S. since it has not been regulated and therefore has been sold online and in physical shops. In recent years, there has been speculation that kratom "being sold in the West" may have contaminants or is also being improperly identified and prepared (Singh et al., 2016). The Dietary Supplement Health and Education Act of 1994 established the marketing, labeling and safety requirements for dietary supplements and ingredients in the U.S., but since kratom is classified as a "new dietary ingredient" it does not have to uphold the regulatory standards and requirements of the act (FDA, 2019; Graves et al., 2021). Though the drug is legal in the U.S., it is controlled in several states including Indiana, Tennessee, Vermont, and Wisconsin (Singh et al., 2016).

A description of kratom usage at the national level was accomplished by Shimmel et al. when they completed a "cross-sectional survey of non-medical use of prescription drugs" from the third quarter of 2018 and first 2019 quarter. The online survey had 59,714 respondents which was weighted to represent the U.S. adult population at the time (252,063,800 people). The kratom usage over the 12-month span was 0.8% or 2,031,803 adults. The lifetime prevalence of kratom usage was 1.3% or 3.3 million. It was found that the majority of kratom users tended to already have "serious substance abuse profiles" (Schimmel et al., 2021). The 12-month span of kratom usage was similar to lysergic acid diethylamide (LSD) and was greater than individual

heroin and methamphetamine usage in the tested timeframe. There were limitations to this study since it was only representative of two quarters of time between 2018 and 2019 and the survey was only completed by 59,714 people (Schimmel et al., 2021).

Kratom use in the U.S. has steadily increased year over year. According to the National Forensic Laboratory Information System, seizures of the drug in the U.S. have been on the rise since 2010 (S. Basiliere & Kerrigan, 2020a; Cinosi et al., 2015). Reports of kratom poisonings within a 12 month timeframe went from 1 report in 2010 to 589 reports in 2018 (DEA, 2019). A commercial toxicology testing laboratory in the U.S., National Medical Services (NMS) Labs, reported that from 2017 to 2018 there was a 53% increase in total mitragynine cases analyzed at their laboratory (NMS Labs, 2021). In 2009, de Moraes et al. concluded that because the sales of kratom were increasing, the amount of research into the pharmacology needed to increase as well (de Moraes, Moretti, Furr, McCurdy, & Lanchote, 2009). Anwar et al. suggested that since the usage of kratom in the U.S. is rising, the reported medical outcomes and health effects could lead to an emerging public health threat (Anwar et al., 2016).

Pharmacodynamics/Pharmacokinetics

M. speciosa contains over 40 alkaloids including mitragynine, paynantheine, speciogynine, and 7-OH. The pharmacokinetics of the plant are considered "complex" owing to its many alkaloids, but the most researched alkaloids include mitragynine and 7-OH (S. P. Basiliere, 2019). Mitragynine and 7-OH are the more heavily researched because mitragynine is the most "abundant active alkaloid" in kratom and 7-OH has more affinity for the μ-opioid receptor because of its hydroxyl group at C-7 (Cinosi et al., 2015; Matsumoto et al., 2004; Trakulsrichai et al., 2015).

The typical route of administration for kratom is orally as tea, tablets, capsules, or chewed leaves. The effects of the drug have been reported to occur 5 to 10 minutes after ingestion and can last between 2 to 5 hours according to the DEA (DEA, 2019). The oral bioavailability of mitragynine is estimated to be 21% which means that only a small fraction of the drug makes it to systemic circulation unchanged (Ya et al., 2019). After oral administration, mitragynine is quickly absorbed in the small intestine since it is a weak base, which is shown by its T_{max}, which is the time it takes for the drug to reach maximum concentration, is 1.5 hours (de Moraes et al., 2009; Ya et al., 2019). Parthasarathy, on the other hand, reported that the oral absorption of mitragynine was "slow, prolonged and incomplete" (Parthasarathy et al., 2010; Ya et al., 2019). The difference in T_{max} from Parthasarathy et al. has been assumed to be a result of late sampling or because the rats were sampled from the tail vein (Ya et al., 2019).

The volume of distribution (V_d), or the total amount of drug in the body divided by the concentration in plasma, of mitragynine was evaluated by Trakulsrichai et al. by using chronic kratom users. The larger a Vd, the more a drug tends to leave the plasma and move into tissues of the body. The V_d from their nine subjects was 38.04 ± 24.32 L/kg leading to the conclusion that mitragynine is lipophilic and highly distributed to tissues in the body (Trakulsrichai et al., 2015). Ya also reported a large V_d after their experiment with a range between 37-90 L/kg and a half-life of 3-9 hours (Ya et al., 2019).

Metabolism of mitragynine occurs mostly in the liver according to Trakulsrichai et al. Mitragynine goes through phase I and II metabolism and is extensively metabolized which results in only a small amount of unchanged mitragynine being excreted in the urine (Trakulsrichai et al., 2015; Ya et al., 2019). Phase I metabolism of mitragynine "involves the hydrolysis of the methylester group at C16, demethylation of the methoxy groups at C9 and C17,

then oxidative and reductive transformations which later produce carboxylic acid and alcohol derivatives"(S. P. Basiliere, 2019). Phase II metabolism of mitragynine is extensive and produces a myriad of glucuronide and sulfate conjugates (S. P. Basiliere, 2019).

The phase I metabolites of mitragynine have been described using *in vitro* and postmortem samples and are depicted in Figure 4. Basiliere and Kerrigan described 7-OH, 9-O-demethylmitragynine, 9-O-demethyl-16-carboxymitragynine, and 16-carboxymitragynine as the major metabolites of mitragynine depicted in Figure 4 (S. Basiliere & Kerrigan, 2020a). 9-O-demethylmitragynine was found to be the most abundant metabolite after Phase 1 metabolism in the *in vitro* and human urine samples. 9-O-demethyl-16-carboxymitragynine and 7-OH were also prominent metabolites but 7-OH is more important because of its "pharmacodynamic implications". Basiliere and Kerrigan also comment that additional pharmacokinetic studies using both mitragynine and 7-OH were "warranted" after the completion of their study (S. Basiliere & Kerrigan, 2020a).

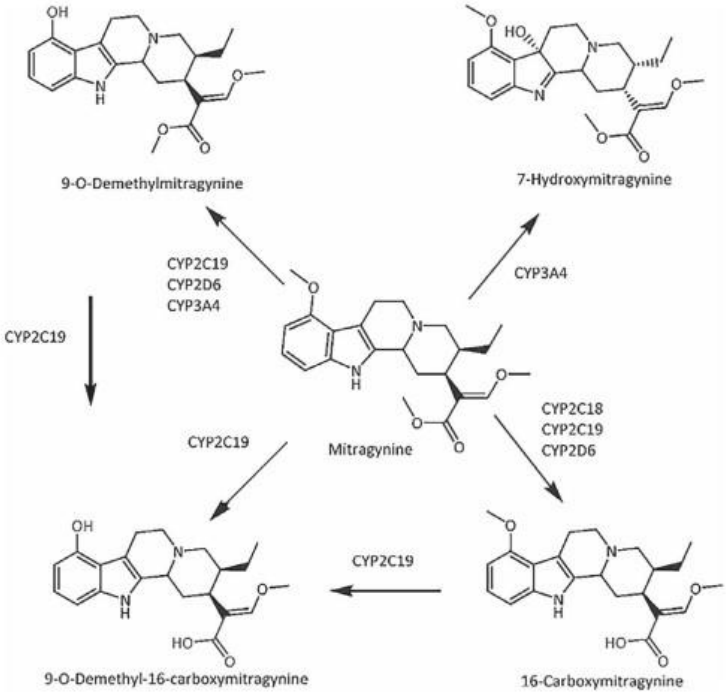


Figure 4. Phase 1 metabolites of mitragynine mediated by CYP450 metabolism (S. Basiliere & Kerrigan, 2020).

Additional *in vitro* experiments by Hanapi, et al. reported that cytochrome P450 (CYP) enzymes 3A4, 2D6, and 2C9 are inhibited by mitragynine. CYP 1A2, on the other hand, was reported to be induced by mitragynine (Eastlack, Cornett, & Kaye, 2020; N A Hanapi et al., 2013). Warner et al. explained that kratom's inhibition or induction of these CYP enzymes had the potential to have "clinically significant interactions" with other drugs which use the same enzymes as substrates (Warner et al., 2016).

Coadministration of mitragynine with other drugs may lead to adverse effects since it interacts with many common CYP enzymes. Kratom products have been reported to be adulterated with other drugs including carisoprodol, modafinil, fentanyl, diphenhydramine, caffeine, and O-desmethyltramadol which have resulted in fatalities (Warner et al., 2016). It has been suggested that fatalities could be caused by herbal-drug interactions when mitragynine is co-administered with other drugs since they are metabolized by the same enzymes (N A Hanapi et al., 2013; Trakulsrichai et al., 2015; Ya et al., 2019). The National Center for Complementary and Integrative Health (NCCIH) reported that most herb-drug interactions identified are hypothetical and not based on rigorous research. NCCIH stated that herb-drug interactions should be monitored in instances of drugs with a narrow therapeutic index, which is the difference in blood concentration between therapeutic and toxic (NCCIH, 2021).

Mitragynine is categorized as a partial or selective agonist of μ-opioid receptors. 7-OH is a full agonist of μ-opioid receptors, therefore it is more potent than mitragynine. Mitragynine is also a competitive antagonist at κ- and δ-opioid receptors (S. Basiliere & Kerrigan, 2020a; Warner et al., 2016; Ya et al., 2019). Mitragynine's activity on μ- and δ-receptors has been identified as the reason for its distinctive analgesic effects (Warner et al., 2016). Mitragynine is considered an "atypical opioid" because it has activity at both opioid and non-opioid receptors

such as monoamine transporter receptors (S. Basiliere & Kerrigan, 2020a). According to Kerrigan & Basiliere, there have been studies that suggest mitragynine causes the restoration of serotonin, norepinephrine, and dopamine levels in the body, which results in an antidepressant effect (Kerrigan & Basiliere, 2022).

Mitragynine has documented antinociceptive, analgesic, anti-inflammatory, antitussive, euphoric, and appetite suppressing effects (S.P. Basiliere, 2019; Veltri & Grundmann, 2019). Ya et al. also reports ileal relaxing and gastric relaxing effects. Side effects of mitragynine have been well documented and usually include dry mouth, anorexia, dehydration, weight loss, hyperpigmentation, constipation and psychosis (DEA, 2019; Trakulsrichai et al., 2015; Ya et al., 2019). Despite the present research of mitragynine, many questions about the compound remain unanswered. Ya et al. stated that knowledge concerning redistribution of the compound to organs over time, potential interactions, bioavailability issues, and the metabolism of mitragynine is inadequate and more exploration is needed (Ya et al., 2019).

Analytical Testing

In the past, identification of kratom alkaloids has been accomplished using many different extractions, matrices, instrumentation, and internal standards. The compounds in *M. speciosa* have been extracted using liquid-liquid extraction (LLE) or solid phase extraction (SPE) methods (Kerrigan & Basiliere, 2022). SPE benefits were described by Parthasarathy as improved selectivity, specificity, higher yield, and increased removal of interferences and particulates (Parthasarathy et al., 2010). LLE, on the other hand, is cost effective, simple, and widely available. The two extraction methods have been utilized in successfully validated methods for mitragynine (S. P. Basiliere, 2019; Le, Goggin, & Janis, 2012; Parthasarathy et al., 2010).

The majority of extractions in the literature for kratom alkaloids have been from a plant matrix (Chittrakarn, Sawangjaroen, Prasettho, Janchawee, & Keawpradub, 2008). Mitragynine and 7-OH have also been identified in biological matrices in the literature including plasma, blood, urine, tissues, and vitreous humor (Arndt et al., 2011; de Moraes et al., 2009; Domingo et al., 2017; Kerrigan & Basiliere, 2022; Mata & Andera, 2020). The selection of instrumentation has been debated for the identification of mitragynine between gas chromatography coupled with mass spectrophotometry (GC-MS) and liquid chromatography coupled with tandem mass spectrophotometry (LC/MS-MS).

In the literature, the use of LC/MS-MS continues to be preferred over GC-MS. The analysis of mitragynine and 7-OH using GC-MS was appealing because it historically was a staple in forensic analysis, but it has been found to be inadequate in resolution of diastereomers and overall lacks sensitivity. Derivatization would also most likely be needed to "overcome poor chromatographic resolution" (Kerrigan & Basiliere, 2022; Wang et al., 2014). According to Kerrigan and Basiliere, GC-MS is also not as useful when examining challenging drugs and polar compounds (Kerrigan & Basiliere, 2022).

LC/MS-MS, on the other hand, has proven to be a viable method for analysis of *Mitragyna* alkaloids. With LC techniques, derivatization is not needed and it provides improved sensitivity and selectivity when mass spectrometry is added (Kerrigan & Basiliere, 2022; Parthasarathy et al., 2010; Wang et al., 2014). De Moraes et al. described the first method which used LC/MS-MS in order to detect mitragynine in rat plasma, and it was described as precise, accurate and highly sensitive (de Moraes et al., 2009). Methods detecting mitragynine were prioritized in the literature, but over time other alkaloids and diastereomers of mitragynine were further

characterized (Arndt et al., 2011; Domingo et al., 2017; Kerrigan & Basiliere, 2022; Philipp et al., 2010).

Several different internal standards have been utilized in the detection of mitragynine and other kratom alkaloids. The most prevalent are deuterated mitragynine and deuterated 7-OH. The use of deuterated internal standards is considered "best practice" as they are close in structure and molecular weight to the undeuterated compound. Therefore, they have similar extraction recovery, ionization response, and chromatographic retention time (Davison, Milan, & Dutton, 2013). Deuterated internal standards are the most prevalent but other internal standards have been utilized in the literature including mefloquine by Parthasarathy et al. and amitriptyline by De Moraes et al. (de Moraes et al., 2009; Parthasarathy et al., 2010).

Le et al. published a qualitative study on 7-OH in human urine using LC/MS-MS that reported 7-OH was found in all urine samples which also contained mitragynine (Le et al., 2012). 7-OH is a metabolite of mitragynine when metabolized by CYP3A4 and is found in the plant material in very small amounts (0.05% of dry plant material) (Kerrigan & Basiliere, 2022; Kruegel et al., 2019). The identification of 7-OH has been preferred along with mitragynine due to its affinity for the μ-opioid receptor. However, one of the problems with quantification of 7-OH is its poor stability in biological samples (S. Basiliere & Kerrigan, 2020a). Another problem encountered in 7-OH identification is the formation of a water adduct when using electrospray ionization when using formic acid as a mobile phase additive (Kerrigan & Basiliere, 2022; Wang et al., 2014).

Identification of kratom alkaloids, such as mitragynine and 7-OH, poses many problems in forensic settings. Kratom is an herbal product, so the separation of mitragynine from diastereomers and other alkaloids must be achieved in order to create an appropriate analytical

method (Papsun et al., 2019; Wang et al., 2014). Pure analytical standards are also needed to differentiate the diastereomers which have until recently been unavailable for purchase. The analytical standards for metabolites are not commercially available except for 7-OH. According to Kerrigan and Basiliere, challenges in the identification of kratom alkaloids need to be considered when developing methods for forensic use (Kerrigan & Basiliere, 2022; Papsun et al., 2019).

Postmortem Testing

The identification of mitragynine has historically been achieved using the plant material. Kerrigan & Basiliere state that forensic identification of mitragynine is not as widespread since most forensic labs do not routinely look for mitragynine or 7-OH in their usual screening methods (immunoassay). Therefore, mitragynine cases are under reported in forensic testing (Kerrigan & Basiliere, 2022).

NMS Labs, which is a private laboratory conducting forensic testing, released an infographic from a study completed by a cross-organizational team from the lab. They stated that mitragynine has increasingly been identified in toxicology casework including driving under the influence of drug (DUID) and medicolegal death investigations (NMS Labs, 2021). In the DUID cases, the concentrations of mitragynine ranged from 11-490 ng/mL in blood. At these concentrations, the police noted swerving, pinpoint pupils and poor motor functions (NMS Labs, 2021). Prosecutors typically attempt to use a drug concentration in blood and associate that concentration with a level of impairment. This presently cannot be done with mitragynine as there is a lack of data associated with antemortem blood concentrations and psychomotor impairment.

The infographic indicated postmortem concentrations less than 500 ng/mL in 81% of cases and less than 1000 ng/mL in 92% of cases (NMS Labs, 2021; Papsun et al., 2019). NMS Labs stated that information comparing the antemortem dosage of mitragynine to the postmortem blood concentration is not yet available. The study completed by NMS Labs and Papsun et al. was intended to highlight the need for unbiased data points as well as case history and comprehensive drug screenings in order to start answering the question of which antemortem dosages lead to fatal postmortem blood concentrations (NMS Labs, 2021; Papsun et al., 2019).

PMR is a phenomenon that has been described for several decades in forensic toxicology. It is the postmortem movement of drugs from areas of high concentration (e.g. liver, lungs, heart) to areas of low concentration (e.g. blood). The shifts in concentration in the postmortem blood may be higher than they were prior to death leading to potential misinterpretation (Shanks, 2021). The mechanisms for this shift after death are drug dependent and multifactorial.

There are trends in the postmortem concentrations which aid in interpretation and help to indicate if a certain drug is more likely to exhibit PMR (I. McIntyre & Escott, 2012). Drug properties such as V_d, protein binding and pKa are stated to be associated with PMR. For example, basic, lipophilic drugs with high V_d are assumed to experience large changes in blood drug concentration after death. McIntyre states that in order to compensate for PMR, postmortem blood specimens should be collected from at least two areas of the body (central and peripheral) so that a comparison in concentrations can be made (I. McIntyre & Escott, 2012).

Several publications covering case studies and associated mitragynine concentrations have been released in recent years (Domingo et al., 2017; Karinen et al., 2014; Mata & Andera, 2020; I. M. McIntyre et al., 2015). McIntyre et al. published a case report on a kratom related fatality of a 24-year-old male in 2015. Mitragynine was identified in the man's system following an

alkaline drug screen using GC-MS. The mitragynine was then quantitated using GC-MS selective ion monitoring analysis. Concentrations for peripheral (0.23 mg/L) and central blood (0.19 mg/L), liver (0.43 mg/kg), vitreous (<0.05 mg/L) and urine (0.37 mg/L) were determined. The case also included therapeutic concentrations of venlafaxine, diphenhydramine, and mirtazapine. The cause of death was ruled to be due to mixed drug intoxication, primarily caused by mitragynine. It has been found that most of the cases which describe mitragynine in the literature include other medications (I. M. McIntyre et al., 2015).

McIntyre et al. also reported a central blood to peripheral blood ratio (C/P) of 0.83 and a liver to peripheral blood ratio of 1.9. It was stated that these ratios suggest no "potential for mitragynine postmortem redistribution (PMR)". The "model" C/P ratio <1.0 and L/P ratio <5 L/kg indicate "little to no propensity toward PMR" (I. M. McIntyre et al., 2015). McIntyre et al. also states that one plot point should be "viewed with caution" and it is implied that more testing is needed to determine the potential for mitragynine to undergo PMR (I. M. McIntyre et al., 2015).

Mata et al. also published several case studies which included mitragynine concentrations. The paper describes 20 death investigations between 2017 and 2018 in Orange County, California, in which mitragynine was identified along with other drugs in some cases. The range of mitragynine concentrations in central blood was 10-4,310 ng/mL with a mean of 626 ng/mL. The peripheral blood had a range of 24.6-3,420 ng/mL with a mean of 903 ng/mL. The C/P ratios in cases with only mitragynine in the cause of death were 0.79, 0.92, and 1.26. The C/P ratios for cases with mitragynine contributing to other drugs in the cause of death were 0.04, 0.37, 0.50, 0.72, 0.87, 0.90, 1.03, and 1.21 (Mata & Andera, 2020).

It was determined that in the 13 cases which analyzed the peripheral and central blood concentrations, mitragynine was not prone to PMR. 7-OH was not quantified in their study but was screened for in 10 of the 20 cases. Only two cases detected 7-OH and there was not a significant correlation between mitragynine concentration and 7-OH detection.

An additional case study in the literature was prepared by Karinen et al. in 2014. They studied an accidental poisoning that they determined was caused in majority by mitragynine. Other drugs including zopiclone, citalopram, and lamotrigine were identified in therapeutic concentrations. The concentration of mitragynine (1.06 mg/L) and 7-OH (0.15 mg/L) were determined in blood. In the urine, the concentration of mitragynine (3.47 mg/L) and 7-OH (2.20 mg/L) were discovered to be higher than in the blood. They determined that the concentrations of mitragynine and 7-OH were large enough to rule cause of death as intoxication. Karinen et al. also recommended that mitragynine and 7-OH be analyzed in postmortem cases which include suspected kratom intoxication (Karinen et al., 2014).

Domingo et al. in 2017 presented two fatalities in which mitragynine was identified. In one of the cases, the mitragynine concentration was 790 ng/mL but the cause of death was the aspiration of chyme (Domingo et al., 2017). There were also several other drugs present in the decedent including etizolam, triazolam, fluoxetine, pregabalin, pipamperone, lorazepam, quetiapine, and olanzapine. It was proposed that the decedent consumed a large amount of kratom preceding death, causing the large mitragynine concentration in the blood. The other case that was studied only had a mitragynine concentration of 10 ng/mL and died from a mixed intoxication with heroin, methamphetamine, MDMA, and GHB. (Domingo et al., 2017). The study also found that the diastereomers of mitragynine appeared in large amounts, but they were not quantitated. Those authors chose not to identify 7-OH in their study because the main focus

was to obtain more information on mitragynine concentrations in fatalities. Domingo et al. also stated that more detailed investigations into mitragynine toxicity, postmortem chemical stability, and mechanisms and amounts of different mitragynine alkaloids in the plant and metabolites are needed to make reasonable interpretations in lethal cases (Domingo et al., 2017).

Stability

The stability of mitragynine and 7-OH have been prioritized over the other kratom alkaloids in the literature (S. P. Basiliere, 2019). Mitragynine stability has mostly been studied as a part of method validations in both blood and serum. Papsun et al. also observed the stability of mitragynine in blood. Aliquots were stored at frozen (-70 and -20℃), refrigerated (4℃), and room temperature (25℃) conditions in purchased sodium fluoride/potassium oxalate preserved blank human whole blood. It was found that mitragynine in blood is stable up to 30 days when refrigerated or frozen with a loss less than 20%. Between 30 and 90 days, mitragynine experienced a loss of greater than 20% at all temperatures. Papsun et al. determined that mitragynine stability in blood declines markedly after 30 days; therefore, the longer the period between specimen collection and testing will result in a larger decrease in the original concentration (Papsun et al., 2019).

Parthasarathy et al. in 2010 also studied the stability of mitragynine in plasma. They stated after three freeze (-20℃) and thaw (25℃) cycles, mitragynine was stable. Short-term stability was tested for 4 hours at room temperature (25℃), as well as after processing stability (12℃) for 16 hours which both proved to be stable (Parthasarathy et al., 2010).

Temperature has large effects on stability, but pH also plays a large role in the stability of a drug as well. Mitragynine has been found to be unstable in highly acidic conditions, shown by its

26% decrease in concentration in simulated gastric fluid (pH 1.2). On the other hand, in simulated intestinal fluid (pH 6.8), it only decreased by 3.6% (Kerrigan & Basiliere, 2022; Manda et al., 2014). Ya et al. in 2019 reported that at 37 ℃, mitragynine was "moderately" stable at the pH of 7 and had a 3.5% decrease after 3 hours. At a pH of 1.2, on the other hand, mitragynine degraded 26% after 1-2 hours. The large decrease in concentration in highly acid media indicates that mitragynine is acid labile. Kerrigan and Basiliere stated that since strongly acidic or basic conditions are used in extractions and higher temperatures can be used in evaporation steps, the stability of mitragynine must be considered (Kerrigan & Basiliere, 2022).

The stability of mitragynine (MG), 7-hydroxymitragynine (7-MG-OH), speciociliatine (SC), speciogynine (SG), and paynantheine (PY) was determined at several different pH levels as well as different temperatures in a study conducted by Basiliere et al. in 2019 (S. P. Basiliere, 2019). A table of the mean percent loss at 8 hours for each alkaloid at each pH and temperature is represented by Table 1 (S. P. Basiliere, 2019). The table depicts a large loss of concentration for mitragynine at pH 2, 8 and 10 at temperatures over 40℃. 7-OH has large losses of concentration at all pH levels over the temperature of 40℃. The alkaloids have significant losses at pH 2 and temperatures above 60℃.

Drug	pH	Mean % Loss at 8 hours				
		80°C	60°C	40°C	20°C	4°C
MG	2	**100**	**47**	0	1	0
	4	0	0	0	1	0
	6	0	7	0	8	0
	8	**31**	13	20	1	0
	10	**25**	**23**	12	18	0
7-MG-OH	2	**100**	**93**	**32**	4	0
	4	**93**	**35**	7	9	0
	6	**65**	**44**	19	0	0
	8	**57**	**32**	9	0	0
	10	**66**	**46**	8	7	0
SC	2	**58**	17	12	-	-
	4	0	0	0	-	-
	6	0	5	11	-	-
	8	20	2	11	-	-
	10	20	7	6	-	-
SG	2	**43**	5	6	-	-
	4	0	0	0	-	-
	6	0	6	11	-	-
	8	11	2	11	-	-
	10	15	3	4	-	-
PY	2	**37**	4	6	-	-
	4	0	0	0	-	-
	6	0	1	9	-	-
	8	5	0	7	-	-
	10	8	3	2	-	-

Table 1. Stability of MG, 7-OH, SC, SG, and PY over 8 hours (S. P. Basiliere, 2019).

The data collected by Basiliere et al. confirms that mitragynine has stability issues at higher temperatures and high and very low pH levels. It also confirms that 7-OH is the most unstable of all the alkaloids tested and is very unstable at all pH levels and all temperatures above 40℃. In simulated gastric fluid, 7-OH concentration decreased by 27% and its decrease corresponded to the increase of mitragynine (Kerrigan & Basiliere, 2022; Manda et al., 2014). Kerrigan & Basiliere stated in their review in 2022 that no literature had addressed the long-term stability of kratom alkaloids in the biological matrices which are commonly encountered in forensic toxicology investigations (Kerrigan & Basiliere, 2022).

Summary

Kratom has a rich ethnomedicinal history which dates back centuries in Southeast Asia, where it became a popular opiate substitute, fatigue reliever, and for socioreligious purposes. The plant has over 40 alkaloids that have been identified over the last century. Mitragynine and 7-OH have been researched because of their abundance and potency, respectively. Kratom is banned in several countries because of its psychoactive effects.

The prevalence of mitragynine has rapidly increased in the United States over the last decade, with an over 50% increase in mitragynine cases between 2017-2018 completed by NMS Labs. The drug is legal to buy online and from head shops across the United States. Mitragynine is a basic, lipophilic drug which is metabolized extensively in the body. It is a partial μ-opioid receptor agonist and 7-OH is a full agonist. Mitragynine also has moderate and non-selective binding affinities at several adrenergic receptors (e.g. α1, α2). 7-hydroxymitragynine, on the other hand, has little to no binding affinity to adrenergic receptors (Nur Aziah Hanapi, Chear, Azizi, & Yusof, 2021). Mitragynine is often used alongside other drugs, which has been a cause for concern since it inhibits several prominent CYP enzymes. Additive or synergistic effects of mitragynine with other medications are a concern.

Analytical methods for mitragynine include different extractions, instrumentation, and internal standards. LC/MS-MS has been used most often in the literature because it can differentiate the botanical alkaloids and diastereomers for kratom and it is overall more sensitive and selective. SPE and LLE extractions have been validated in the literature with pros and cons for both methods. There are several difficulties in validating a method for mitragynine and/or 7-OH because of the diastereomers that must be differentiated and the stability of 7-OH in biological matrices over time.

Forensic analysis of mitragynine is less available because most forensic laboratories are not searching for the drug in their screening methods. Multiple publications have completed case studies for mitragynine with several attributing mitragynine as the main contributor to cause of death. The toxic and lethal concentrations of mitragynine have not been well determined and the PMR for the drug has little research. These lead to issues in interpretation when determining the overall cause of death, especially when mitragynine is found with other drugs. 7-OH has not been researched heavily in a postmortem setting because it is found in such small amounts in the plant material, postmortem samples, and has poor stability.

The stability of mitragynine, 7-OH and other alkaloids has not been widely researched. Within method validations, mitragynine has been tested for stability in blood and serum in short-term studies. In long-term studies, it has been found that mitragynine is not stable in pH levels 2, 8, 10 and at temperatures over 40℃. 7-OH is even less stable and has massive losses in concentration at all pH levels when the temperature is over 40℃. Stability must be assessed when determining analytical methods to be validated since high or low pH levels and high temperatures can be found during extraction and evaporation steps respectively.

The research on kratom has revealed that it has a high therapeutic and abuse potential. Additional research is still needed to understand the extent of these potentials. A potential public health threat is forming because of the lack of epidemiological data for kratom. Forensic labs do not routinely search for the drug; therefore, the amount of people taking mitragynine and the exact dosage remains unknown. Additional forensic testing is needed to determine the potential for the drug to undergo PMR and the toxic and lethal concentrations associated with mitragynine.

Materials and Methods

Method Development

The sample preparation technique, or extraction, was evaluated and optimized by the OCME laboratory prior to finalizing the method validation plan. An alkaline extraction using ammonium hydroxide followed by a cleanup step with hexanes was first attempted. It was found during that development step that mitragynine converted to 7-OH in the basic conditions of the extraction. Upon learning of the conversion, an acetonitrile "crash" procedure was tested which utilized the addition of cold acetonitrile to the sample while vortexing followed by a cleanup with hexanes. A crash can be useful because it is inexpensive and causes proteins which interfere with an analysis to fall out of solution. The crash procedure did not result in the loss of target analyte, but it did not produce good response on the LC/MS-MS. A supported liquid extraction (SLE) using 3 mL columns containing diatomaceous earth to separate the target analytes from the biological matrix was then utilized. The added SLE aspect helped to further clean the extract; thus, it was selected as the method to utilize for method validation. The SLE method was evaluated and optimized in postmortem samples prior to full implementation of the extraction method in the validation plan.

In accordance with the American Academy of Forensic Sciences Standards Board (ASB) standards, the method validation for the main analyte, mitragynine, included studies for interferences (endogenous matrix interferences and interferences from other commonly encountered analytes), ion suppression/enhancement, limit of detection, calibration model, bias, precision, carryover and processed stability in blood and liver. A qualitative method for 7-OH was validated, and the method validation assessed interferences, ion suppression/enhancement, limit of detection, carryover, and processed stability in blood and liver. Robustness of the

mitragynine method was also assessed by analyzing the calibration on a secondary LC/MS-MS and by an additional analyst.

Reagents and Standards

The solvents used were HPLC grade or better. Pierce™ acetonitrile (LC-MS Grade) was purchased from Thermo Scientific (Waltham, MA), while Optima LC/MS-grade formic acid and methylene chloride were purchased from Fisher Chemical (Hampton, NH). HPLC-grade deionized (DI) water was generated in lab using a Millipore Direct-Q® 3UV (Burlington, MA) water purification system, resulting in >18.0 MΩ·cm filtered deionized water (DI). ACS-grade sodium phosphate dibasic anhydrous and potassium phosphate monobasic were purchased from Fisher Scientific (Hampton, NH) for the phosphate buffer preparation. Hexanes was purchased from Fisher Chemical (Hampton, NH) for later clean-up steps. Syringe filters (Titan 0.2 μm, 4 mm) and syringes (1 mL) were purchased from Thermo Scientific (Waltham, MA) and utilized at the end of the extraction to filter the aliquots before running them on the instrument.

Reference materials of 100 μg/mL of 7-OH, mitragynine-d3, and 7-OH-d3 in methanol were purchased from Cerilliant Corporation (Round Rock, TX). A reference material of 1.0 mg/mL of mitragynine in methanol was purchased from Cayman Chemical (Ann Arbor, MI).

The 0.5 M phosphate buffer, pH 5.5, was prepared by dissolving 68 g KH_2PO_4 in DI water and bringing it to 1.0 L for solution A. Solution B was prepared by dissolving 17.7 g Na_2HPO_4 in DI water and bringing it up to 250 mL. Solution A's pH was adjusted to 5.5 by adding small amounts of solution B until the correct pH was attained. Mobile phase A (0.1% formic acid in DI water) was prepared by adding 500 μL of concentrated formic acid to 500 mL of DI water. Mobile Phase B (0.1% formic acid in acetonitrile) was prepared by adding 500 μL of formic acid to 500 mL of acetonitrile. The reconstitution solvent was prepared by adding 90 mL of mobile

phase A to 10 mL of mobile phase B. Preparation of the saturated hexane wash was completed by adding hexanes to reconstitution solvent in a 4:1 ratio (i.e. 200 mL hexanes: 50 mL reconstitution solvent).

Specimen collection

Expired packed red blood cells (RBC) were purchased by the OCME laboratory from the Oklahoma Blood Institute (OBI) and used as "control blood" throughout the project. The control blood was prepared by adding the RBC to a 500 mL container, adding 10 g sodium fluoride and 100 mL saline solution then mixing. The control blood was screened by routine toxicological analyses including immunoassay (ELISA) drug screen, alkaline drug screen, and acid-neutral drug screen to rule out possible contamination. The blood was stored in refrigerated conditions (4^{o}C) until needed.

The stability study used control blood as the matrix. Ten milliliters of control blood was fortified with 100 µL of 100 µg/mL 7-OH resulting in 1 µg/mL. The 10 mL was aliquoted into separate vials and put in the three different storage conditions including room temperature (21°C), refrigerated (4°C), and frozen (-30°C). A low and high concentration of mitragynine were tested at the three storage conditions. The low concentration of mitragynine was prepared by fortifying 15 µL of 100 µg/mL mitragynine reference material solution into 10 mL of control blood, resulting in a concentration of 150 ng/mL. The high concentration of mitragynine was prepared by fortifying 130 µL of 100 µg/mL mitragynine reference material solution into 10 mL of control blood, resulting in 1300 ng/mL. Five hundred microliters of each concentration of mitragynine were aliquoted into individual vials and placed at the three storage conditions for the stability study.

The method validation used de-identified, drug free (blank) postmortem blood and liver samples. The specimens were chosen using the criteria of a negative ELISA and alkaline drug screen. Case study samples were selected using the criteria of a presumptive positive of mitragynine from the alkaline drug screen. De-identified heart blood, femoral blood, and liver samples were used to quantitate mitragynine and qualitatively identify 7-OH. The liver samples were prepared by weighing out 5 grams of liver and then diluting 1:4 with DI water. The dilution was then homogenized using a bead ruptor homogenizer (OMNI International, Kennesaw, GA).

Preparation of working solutions

The working solutions were prepared for a calibration curve extending from 5 ng/0.1 mL to 160 ng/0.1 mL. A working solution (1 μg/mL) of the deuterated internal standards (mitragynine-d3 and 7-OH-d3) was prepared in methanol from their original 100 μg/mL certified reference material (CRM) concentrations. A calibration curve including six points was prepared using 5, 10, 20, 40, 80, and 160 ng/0.1 mL. Three different quality controls (QC) including low (15 ng), medium (50 ng), and high (130 ng) concentrations were prepared using a 100 μg/mL certified reference material solution of mitragynine. A single point methanolic calibrator for 7-hydroxymitragynine was prepared at a concentration of 0.4 mcg/mL using certified reference material. During analysis, 0.1 mL control blood was fortified with 25 μL of the 0.4 mcg/mL solution resulting in a calibrator concentration of 10 ng/0.1 mL.

Sample preparation and extraction

Analysis of samples included pipetting 25 μL of the 1 μg/mL internal standards into the bottom of small disposable test tubes. Then 25 μL of each calibrator was pipetted into the bottom of its respective tube. The mitragynine quality controls were pipetted (25μL) into their respective tubes, then the 7-OH calibrator was pipetted (25 μL) into its respective tube. Phosphate buffer

(400 µL at 0.5 M) was added into each tube and then vortexed well. Sample or control blood was then pipetted (0.1 mL) into the appropriate tubes and vortexed.

The samples were then poured onto 3 mL Agilent ChemElut® (diatomaceous earth) partitioning columns, which were positioned on a positive pressure manifold. The samples would then sit for ten minutes. Methylene chloride (2.5 mL) was added to each column and allowed to elute by gravity for ten minutes. Two additional aliquots of methylene chloride were added and low pressure applied on the manifold once the solvent stopped dripping. The eluted solvent was evaporated to dryness under a stream of nitrogen in a heated water bath (40^{o}C). The samples were reconstituted with 200 µL of Mobile Phase A: Mobile Phase B (90:10 (v/v)). Samples were vortexed and 200 µL of saturated hexane wash (4:1 hexanes: reconstitution solvent) was added. Samples were vortexed again, then centrifuged for 5 minutes at 2600 rpm. The top layer was aspirated to waste and the bottom layer was filtered into autosampler vials using 0.2 µm Titan syringe filters.

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC/MS-MS)

The instrumentation utilized in this study was a LC/MS-MS system and involved an Agilent 6420 triple-quadrupole mass spectrometer paired with an Agilent 1290 Infinity series liquid chromatography system. The system consisted of a G4226A auto-sampler, G4204A quaternary pump, and a G1316A thermostat-regulated column compartment.

An Agilent Poroshell 120 EC-C18; 2.7 um; 2.1 x 75 mm high performance liquid chromatography (HPLC) column was used for this study because it is a good choice for a wide pH range and the usage of polar mobile phase. The multiple-reaction monitoring (MRM) method was utilized for this study and three transitions were monitored for 7-OH, mitragynine and

mitragynine-d3, while two transitions were monitored for 7-OH-d3. Fragmentation and instrument conditions are shown in Table 2.

Table 2 Fragmentation and Instrument Conditions

Compound Name	ISTD	Precursor Ion	Product Ion	Retention Time	Fragmentor (V)	CE (V)	Cell Acc (V)	Polarity
7-OH Mitragynine	No	415.2	190.1	2.86	198	30	4	Positive
7-OH Mitragynine	No	415.2	175.1	2.86	198	54	4	Positive
7-OH Mitragynine	No	415.2	146.1	2.86	198	86	4	Positive
Mitragynine	No	399.2	226.1	4.5	134	22	4	Positive
Mitragynine	No	399.2	174	4.5	134	34	4	Positive
Mitragynine	No	399.2	159.0	4.5	134	58	4	Positive
Mitragynine-D3	Yes	402.2	177.1	4.5	152	30	4	Positive
Mitragynine-D3	Yes	402.2	110.1	4.5	152	38	4	Positive
7-OH Mitragynine-D3	Yes	418.2	193.1	2.87	196	34	4	Positive
7-OH Mitragynine-D3	Yes	418.2	175.1	2.87	196	50	4	Positive

Fragmentor voltage is the energy required to fragment the precursor ions into product ions. Collision energy (CE) is the amount of energy that a precursor ion receives as it is accelerated to the collision cell, then colliding with gas molecules and fragmenting. Cell acceleration (Cell Acc) is the electric potential that excels ions through the collision quadrupole.

Instrument Conditions

The run time on the instrument was 8.0 minutes with a 1.40 minute post time. The method used positive electrospray ionization and multiple reaction monitoring scanning mode. Two microliters of the sample were injected onto the column and the flow rate was 0.500 mL/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile, labeled as mobile phase A and B respectively. The temperature of the column compartment was kept at 40^{o}C. The capillary

voltage was set to 400 volts. Table 3 depicts the gradient flow and LC conditions used in this study.

Table 3 Gradient Flow LC Conditions

Time (mins)	Mobile Phase A*	Mobile Phase B**	Stop time (mins)
0.0	90%	10%	8.00
0.05	90%	10%	**Post time (mins)**
6.0	50%	50%	1.40
7.0	10%	90%	
8.0	10%	90%	

*Mobile Phase A: 0.1% formic acid in water
**Mobile Phase B: 0.1% formic acid in acetonitrile

Method Validation

Interferences, bias, precision, ion suppression/enhancement, limit of detection, carryover, calibration model, and stability were assessed in the method validation for mitragynine. 7-OH was validated for qualitative identification and included interferences, ion suppression/enhancement, limit of detection, carryover, and stability.

Interferences

The method should be able to differentiate unequivocally the analytes of interest from the internal standard, from interferences such as endogenous matrix compounds, and from other commonly encountered compounds such as structural analogues and metabolites.

Endogenous Matrix Interference

Blank matrix samples from a minimum of ten different sources without the addition of internal standard were analyzed by the method to demonstrate the absence of common interferences from the matrix. Ten sources of drug free postmortem blood and liver homogenate samples from previously analyzed cases were extracted according to the newly developed method. In addition to these ten samples a quality control sample (low concentration) with internal standard, a 7-OH calibrator (10 ng) with internal standard, and a blank matrix sample were extracted in blood from the local blood bank (control blood) to have a comparison against potential postmortem matrix artifacts that may be present in the postmortem samples.

Interferences from Commonly Seen Compounds

Blank matrix samples that are fortified with other analytes that have the potential to interfere with the method's analytes were evaluated. The most common drugs/metabolites encountered in the OCME laboratory and/or structurally similar compounds were evaluated for interferences. Five multi-drug test mixes (Table 4) that contain the most commonly seen drugs and have potential for interference were extracted with the newly developed method in ten sources of pooled drug free postmortem matrix samples (blood and liver homogenates). A negative control with internal standard sample, a high mitragynine QC sample with no internal standard, and a 7-OH calibrator with no internal standard were also analyzed.

Table 4 Commonly Seen Drugs Used in Interference Study

	*Extracted concentration is 1.25 mcg/mL unless noted otherwise			
Mix #1	Mix #2	Mix #3	Mix #4	Mix #5
Opiates and Related	**Drugs of Abuse**	**Prescription Drugs**	**OTC Drugs**	**Antidepressants**
Codeine	Amphetamine	Metoprolol	Pseudoephedrine	Fluoxetine
Hydrocodone	Methamphetamine	Verapamil	Lidocaine	Venlafaxine
Oxycodone	Phencyclidine	Atropine	Doxylamine	Amitriptyline
Oxymorphone	Cocaine	Hydroxyzine	Dextromethorphan	Doxepin
6-MAM (250 ng)	THC (250 ng)	Quetiapine	Acetaminophen (125 mcg)	Mirtazapine
Morphine	THCCOOH (1.25 mcg)	Zolpidem	Ibuprofen (125 mcg)	Cyclobenzaprine
Hydromorphone	Benzoylecgonine	Butalbital (125 mcg)	Diphenhydramine	Sertraline
Fentanyl (250 ng)		Phenytoin (125 mcg)		Citalopram
Tramadol	&	Phenobarbital (125 mcg)		Trazodone
Methadone	**Benzos**	Carisoprodol		
Buprenorphine (250 ng)	Alprazolam	Lamotrigine		
	7-Aminoclonazepam			
	Diazepam			
	Midazolam			
	Nordiazepam			
	Temazepam			

Bias

Bias was measured in pooled fortified matrix samples using a minimum of three separate samples per concentration at three different concentration levels (low, medium, high) over five different runs. The maximum acceptable bias is ±20% at each concentration. The bias was calculated for each concentration using the following formula:

$$Bias\ (\%)\ at\ concentration = \left[\frac{Grand\ Mean\ of\ Calculated\ Concentration - Nominal\ Concentration}{Nominal\ Concentration}\right] X\ 100$$

To establish the method's bias, blank matrix samples (control blood and pooled liver homogenates) were extracted in triplicate at the following concentrations: low (15 ng); medium (50 ng); and high (130 ng). These concentrations of fortified samples were analyzed on five separate days with a freshly prepared calibration curve.

Precision

Precision, coefficient of variation (%CV), was assessed using three different samples at three different concentration levels (low, medium, and high) over five different runs. Two types of

precision studies were assessed during validation: within run precision and between run precision. The %CV should not exceed 20% at each concentration.

Within run precision is calculated as follows:

$$Within - run\ CV\ (\%) = \left[\frac{Standard\ deviaition\ of\ a\ single\ run\ of\ samples}{Mean\ calculated\ value\ of\ a\ single\ run\ of\ samples}\right] X\ 100$$

Between run precision is calculated as follows:

$$Between - run\ CV\ (\%) = \left[\frac{Standard\ deviaition\ of\ all\ observations\ for\ each\ concentration}{Grand\ mean\ for\ each\ concentration}\right] X100$$

Ion Suppression/Enhancement

Ion suppression/enhancement in LC/MS-MS is a type of matrix effect and can occur when a closely eluted compound either suppresses or enhances the ionization of the analyte molecules in a mass spectrometer's source. Determination of the matrix effect allows the assessment of the reliability and selectivity of an LC/MS-MS method. Post-extraction addition technique was used to evaluate ion suppression and enhancement. A set of neat samples were prepared at two QC concentrations (one low and one high) for mitragynine as well as one 7-OH calibrator. Each of these was injected six times to establish an average peak area for each concentration. For the second set of samples, ten different sources of blank postmortem matrix samples (blood and liver homogenates) were extracted in duplicate according to the newly developed method. After the drying step of the procedure, each sample was then fortified with a low and high concentration QC, or 7-OH calibrator and dried again. The reconstitution of the samples was carried out according to the procedure.

To assess this phenomenon a quality control sample and the internal standard (neat sample-A) should be compared to post-extracted sample matrix fortified with standards and internal

standards at the same concentrations (B). Ten replicates of each should be compared and expressed as a percentage of matrix effect (ME):

$$(ME): ME\ (\%) = B/A \times 100$$

According to ASB standards, average suppression/enhancement should not exceed 25% and the %CV should not exceed 15%. The matrix effect, if observed, should not alter the average standard/internal standard ratio greater than 15% for quantitative assays. For qualitative analysis, while there are no criteria placed on allowable matrix effect, good laboratory practice suggests that the average standard/internal standard ratio should not be greater than 15% and awareness of the effect for any given analyte will be beneficial in assessing whether to report as positive or if the specimen is not suitable for this analysis.

Carryover

To evaluate carryover as part of method validation, blank matrix samples were analyzed immediately after a high concentration sample. The highest analyte concentration at which no analyte carryover is observed, above the method's limit of detection (LOD), in the blank matrix sample is determined to be the concentration at which the method is free from carryover. This concentration was confirmed using triplicate analyses in each matrix.

To assess carryover a sample at the LOD level (1.0 ng/0.1 mL) for mitragynine and 7-OH calibrator (10 ng/0.1 mL) was extracted and injected three times to get an average signal to account for variation between injections. Next, a sample that far exceeds the expected concentration for mitragynine (800 ng/0.1mL) and 7OH (500 ng/0.1mL) in a real case sample was injected followed by an extracted blank blood sample. This process was repeated two more times using the same high concentration sample followed by a new blank each time.

Limit of Detection

Limit of detection represents the lowest analyte concentration for which specific identification criteria can still be fulfilled. The LOD was established using fortified matrix samples from three different sources of blank matrix in duplicate over three runs. LOD samples were prepared for each matrix intended for validation.

Calibration Model

The calibration model is the mathematical model that describes the correlation between the signal response (peak area ratio of analyte and internal standard) and the analyte concentration in the sample. The choice of an appropriate model (i.e., linear or quadratic, unweighted or weighted) is necessary for accurate and reliable quantitative results. Control samples must be prepared in all matrices intended to be analyzed by the method. The working analyte concentration range must include at least six non-zero calibration samples and have five replicates of each concentration established over separate analyses. Once the calibration model has been established during validation the same model must be used during routine analysis.

Stability

Processed sample stability was evaluated by extracting a low (15 ng/0.1mL) and high (130ng/0.1mL) mitragynine QC sample, in duplicate, in blood and liver and a 7-OH (10ng/0.1mL) QC sample and immediately analyzing them on the instrument. Area response was recorded for mitragynine and 7-OH to represent time zero (T_0). Vials with extracted samples remained on the instrument in the autosampler (6°C) and were re-injected at 24, 48, and 72 hours. Area response was recorded for mitragynine and 7-OH for the various time intervals. Analytes were considered stable if the response was within ±20% of T_0 response average, according to ASB standards.

Long-term stability was evaluated at two concentrations (15 ng/0.1 mL and 130 ng/0.1 mL) for mitragynine and at 100 ng/0.1 mL for 7-OH in triplicate, in three different storage conditions – ambient (21°C), refrigerated (4°C), and frozen (-30°C) temperature. Ambient conditions were evaluated at 0, 24, 48, and 72 hours, while refrigerated and frozen samples were evaluated at 7, 14, 21 30, 65, and 90 days. On day zero of the study, three separate 10 mL aliquots of control bloods were fortified with either 100 µL of 7-OH, 15 µL of mitragynine and 130 µL of mitragynine, respectively, yielding a final concentration of 100 ng/0.1mL for 7-OH, 15 ng/0.1mL and 130 ng/0.1mL for mitragynine. The specimens were aliquoted out for the 72-hour ambient study as well as the 90-day refrigerator and freezer condition studies. The aliquots were stored in glass microvials.

Results

Interferences

There were no interferences present in the blood or liver for mitragynine, d3-mitragynine, 7-OH, or d3-7-OH. There was no contribution of analyte to internal standard response, and no contribution of internal standard to analyte response. Several drug classes (Table 4) were analyzed at 1.25 µg/mL. Figure 5 depicts the LC/MS-MS chromatographic data of 15 ng/0.1 mL (low) quality control.

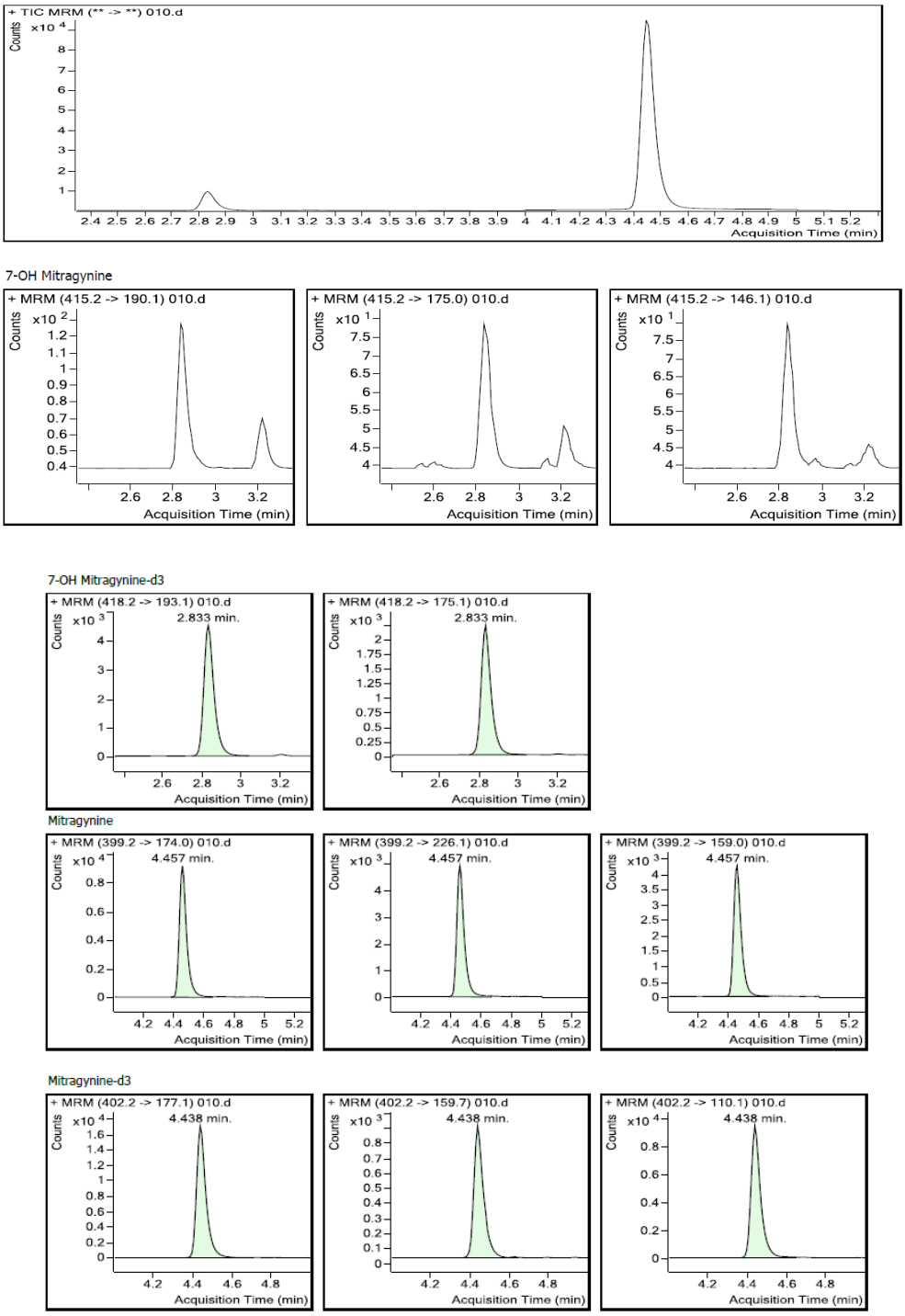


Figure 5. 15 ng/0.1 mL quality control sample with 7-OH-d3 at 2.833 minutes, mitragynine at 4.457 minutes, and mitragynine-d3 at 4.438 minutes.

Bias

Bias was less than 20% across all runs for both blood and liver matrix. Table 5 and Table 6 depict the results for the five calibration curves, along with the mean for each quality control level for blood and liver. Bias calculations using the mean concentration from each concentration level are listed in Table 7.

Table 5 Quality Control Data used to Calculate Bias for Blood

	25 µL = 15 ng		25 µL= 50 ng		25 µL= 130 ng	
Curve Date		**Results**		**Results**		**Results**
10/11/2022		14		47		132
		14		51		125
		15		47		125
10/17/2022		14		50		134
		14		51		133
		12		49		125
10/24/2022		15		56		135
		16		54		126
		15		53		127
10/25/2022		15		50		117
		15		49		126
		15		51		130
10/27/2022		13		46		120
		15		48		116
		15		46		119
	N=	15	**N=**	15	**N=**	15
	Mean	14.47	**Mean**	49.87	**Mean**	126.00
	SD	0.990	**SD**	2.924	**SD**	6.071
	CV (%)	6.85%	**CV (%)**	5.86%	**CV (%)**	4.82%

Table 6 Quality Control Data used to Calculate Bias for Liver

	25 µL = 15 ng		25 µL= 50 ng		25 µL= 130 ng	
Curve Date		**Results**		**Results**		**Results**
10/11/2022		14		48		124
		16		48		133
		15		40		119
10/17/2022		14		50		114
		15		52		118
		16		58		111
10/24/2022		15		50		131
		15		48		128
		15		48		121
10/25/2022		15		50		127
		15		51		123
		14		51		117
10/27/2022		14		47		114
		14		46		117
		13		42		114
	N=	15	**N=**	15	**N=**	15
	Mean	14.67	**Mean**	48.60	**Mean**	120.73
	SD	0.816	**SD**	4.188	**SD**	6.724
	CV (%)	5.57%	**CV (%)**	8.62%	**CV (%)**	5.57%

Table 7 Bias Calculations in Blood and Liver

Mitragynine in Blood		
Target Concentration (ng)	**Mean Concentration (ng)**	**Bias %**
Low - 15	14.5	-3.5
Medium - 50	49.9	-0.3
High - 130	126	-3.1
Mitragynine in Liver		
Target Concentration (ng)	**Mean Concentration (ng)**	**Bias %**
Low - 15	14.7	-2.2
Medium - 50	48.6	-2.8
High - 130	121	-7.1

Precision

Table 8 depicts the quality control data over the five runs used to calculate the within run and between run precision in blood samples. Table 9 contains the data for the quality controls over five runs used to calculate the within run and between run precision in liver samples. Precision (%CV) was less than 20% across all runs for both blood and liver matrix.

Table 8 Mitragynine Within Run and Between Run Precision Results in Blood

Low (15 ng/0.01mL)	Run 1	Run 2	Run 3	Run 4	Run 5
Rep 1	14	14	15	15	13
Rep 2	14	14	16	15	15
Rep 3	15	12	15	15	15
Mean	14.3	13.3	15.3	15.0	14.3
SD	0.6	1.2	0.6	0.0	1.2
CV (%)	4.0%	8.7%	3.8%	0.0%	8.1%
Grand Mean	14.5				
SD	1.0				
Between Run (CV%)	6.8%				
Medium (50 ng/0.01mL)	Run 1	Run 2	Run 3	Run 4	Run 5
Rep 1	47	50	56	50	46
Rep 2	51	51	54	49	48
Rep 3	47	49	53	51	46
Mean	48.3	50.0	54.3	50.0	46.7
SD	2.3	1.0	1.5	1.0	1.2
CV (%)	4.8%	2.0%	2.8%	2.0%	2.5%
Grand Mean	49.9				
SD	2.9				
Between Run (CV%)	5.9%				
High (130 ng/0.01mL)	Run 1	Run 2	Run 3	Run 4	Run 5
Rep 1	132	134	135	117	120
Rep 2	125	133	126	126	116
Rep 3	125	125	127	130	119
Mean	127.3	130.7	129.3	124.3	118.3
SD	4.0	4.9	4.9	6.7	2.1
CV (%)	3.2%	3.8%	3.8%	5.4%	1.8%
Grand Mean	126.0				
SD	6.1				
Between Run (CV%)	4.8%				

Table 9 Mitragynine Within Run and Between Run Precision Results in Liver

Low (15 ng/0.01mL)	Run 1	Run 2	Run 3	Run 4	Run 5
Rep 1	14	14	15	15	14
Rep 2	16	15	15	15	14
Rep 3	15	16	15	14	13
Mean	15.0	15.0	15.0	14.7	13.7
SD	1.0	1.0	0.0	0.6	0.6
CV (%)	6.7%	6.7%	0.0%	3.9%	4.2%
Grand Mean	14.7				
SD	0.8				
Between Run (CV%)	5.6%				
Medium (50 ng/0.01mL)	Run 1	Run 2	Run 3	Run 4	Run 5
Rep 1	48	50	50	50	47
Rep 2	48	52	48	51	46
Rep 3	40	58	48	51	42
Mean	45.3	53.3	48.7	50.7	45.0
SD	4.6	4.2	1.2	0.6	2.6
CV (%)	10.2%	7.8%	2.4%	1.1%	5.9%
Grand Mean	48.6				
SD	4.2				
Between Run (CV%)	8.6%				
High (130 ng/0.01mL)	Run 1	Run 2	Run 3	Run 4	Run 5
Rep 1	124	114	131	127	114
Rep 2	133	118	128	123	117
Rep 3	119	111	121	117	114
Mean	125.3	114.3	126.7	122.3	115.0
SD	7.1	3.5	5.1	5.0	1.7
CV (%)	5.7%	3.1%	4.1%	4.1%	1.5%
Grand Mean	120.7				
SD	6.7				
Between Run (CV%)	5.57%				

Ion Suppression/Enhancement

The raw data shows acceptable chromatography and standard/internal standard ratios are all within 15%. Tables 10-15 depict the ion suppression/enhancement data for mitragynine at the concentration of 15 ng/0.1mL and 130 ng/0.1mL in blood and liver as well as 10 ng/0.1 mL for 7-OH in blood and liver. There was one quantitative ion and two qualitative ions assessed for each analyte. The post extraction abundance is divided by the neat abundance and multiplied by

100 to determine the matrix effect. A number over 100 depicts an enhancement of the ions and a number less than 100 represents a suppression of the ions. A ratio of standard to internal standard abundance was also assessed to make sure that the deuterated internal standard was suppressed or enhanced similarly to the standard.

Table 10 Mitragynine (15 ng/0.1mL) Ion Suppression/Enhancement Data for Quant and Qualifier Ions for Blood

	Mitragynine (399.2/174.0) Quant		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	63637	75643	118.87%
PM Blood 2	63637	80899	127.13%
PM Blood 3	63637	89070	139.97%
PM Blood 4	63637	78251	122.96%
PM Blood 5	63637	80269	126.14%
PM Blood 6	63637	72541	113.99%
PM Blood 7	63637	58151	91.38%
PM Blood 8	63637	67447	105.99%
PM Blood 9	63637	73946	116.20%
PM Blood 10	63637	71262	111.98%
		Average	117.46%
		CV%	10.64%
	Mitragynine (399.2/226.1) Qual1		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	39440	45354	114.99%
PM Blood 2	39440	47725	121.01%
PM Blood 3	39440	51325	130.13%
PM Blood 4	39440	47960	121.60%
PM Blood 5	39440	47129	119.50%
PM Blood 6	39440	44472	112.76%
PM Blood 7	39440	35704	90.53%
PM Blood 8	39440	41156	104.35%
PM Blood 9	39440	43819	111.10%
PM Blood 10	39440	40807	103.47%
		Average	112.94%
		CV%	9.50%

	Mitragynine (399.2/159.0) Qual2		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	28513	33887	118.85%
PM Blood 2	28513	35920	125.98%
PM Blood 3	28513	37417	131.23%
PM Blood 4	28513	34328	120.39%
PM Blood 5	28513	33414	117.19%
PM Blood 6	28513	32328	113.38%
PM Blood 7	28513	27046	94.85%
PM Blood 8	28513	30639	107.46%
PM Blood 9	28513	32281	113.22%
PM Blood 10	28513	33116	116.14%
		Average	115.87%
		CV%	8.16%

Standard/Internal Standard Ratio				
	Standard-Quant	**ISTD-Quant**	**Resp Ratio**	**Deviation**
Neat (15 ng/0.1 mL)	63637	124079	0.5129	0.00%
PM Blood 1	75643	190281	0.3975	-22.49%
PM Blood 2	80899	146731	0.5513	7.50%
PM Blood 3	89070	151646	0.5874	14.52%
PM Blood 4	78251	147564	0.5303	3.39%
PM Blood 5	80269	145949	0.5500	7.23%
PM Blood 6	72541	142078	0.5106	-0.45%
PM Blood 7	58151	124322	0.4677	-8.80%
PM Blood 8	67447	144038	0.4683	-8.70%
PM Blood 9	73946	143462	0.5154	0.50%
PM Blood 10	71262	139085	0.5124	-0.10%
		Average	0.5091	-0.74%

Table 11 Mitragynine (130 ng/0.1mL) Ion Suppression/Enhancement Data for Quant and Qualifier Ions for Blood

	Mitragynine (399.2/174.0) Quant		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	623065	637912	102.38%
PM Blood 2	623065	652371	104.70%
PM Blood 3	623065	661963	106.24%
PM Blood 4	623065	665974	106.89%
PM Blood 5	623065	497881	79.91%
PM Blood 6	623065	631741	101.39%
PM Blood 7	623065	662633	106.35%
PM Blood 8	623065	663228	106.45%
PM Blood 9	623065	594046	95.34%
PM Blood 10	623065	372525	59.79%
		Average	96.94%
		CV%	15.11%

	Mitragynine (399.2/226.1) Qual1		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	378054	383673	101.49%
PM Blood 2	378054	399775	105.75%
PM Blood 3	378054	404266	106.93%
PM Blood 4	378054	397693	105.19%
PM Blood 5	378054	381479	100.91%
PM Blood 6	378054	385105	101.87%
PM Blood 7	378054	394853	104.44%
PM Blood 8	378054	395519	104.62%
PM Blood 9	378054	352331	93.20%
PM Blood 10	378054	223110	59.02%
		Average	98.34%
		CV%	13.85%

	Mitragynine (399.2/159.0) Qual2		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	270324	288096	106.57%
PM Blood 2	270324	293246	108.48%
PM Blood 3	270324	302641	111.95%
PM Blood 4	270324	305249	112.92%
PM Blood 5	270324	280566	103.79%
PM Blood 6	270324	281234	104.04%
PM Blood 7	270324	302129	111.77%
PM Blood 8	270324	289486	107.09%
PM Blood 9	270324	264173	97.72%
PM Blood 10	270324	167165	61.84%
		Average	102.62%
		CV%	13.91%

Standard/Internal Standard Ratio				
	Standard-Quant	**ISTD-Quant**	**Resp Ratio**	**Deviation**
Neat (130 ng/0.1 mL)	623065	136270	4.5723	0.00%
PM Blood 1	637912	139382	4.5767	0.10%
PM Blood 2	652371	131656	4.9551	8.37%
PM Blood 3	661963	145524	4.5488	-0.51%
PM Blood 4	665974	126029	5.2843	15.57%
PM Blood 5	497881	131486	3.7866	-17.18%
PM Blood 6	631741	137298	4.6012	0.63%
PM Blood 7	662633	133957	4.9466	8.19%
PM Blood 8	663228	124955	5.3077	16.09%
PM Blood 9	594046	135774	4.3753	-4.31%
PM Blood 10	372525	85601	4.3519	-4.82%
		Average	4.6734	2.21%

Table 12 Mitragynine (15 ng/0.1mL) Ion Suppression/Enhancement Data for Quant and Qualifier Ions for Liver

	Mitragynine (399.2/174.0) Quant		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Liver 1	76110	84209	110.64%
PM Liver 2	76110	82900	108.92%
PM Liver 3	76110	60367	79.32%
PM Liver 4	76110	73092	96.04%
PM Liver 5	76110	81160	106.64%
PM Liver 6	76110	78071	102.58%
PM Liver 7	76110	53141	69.82%
PM Liver 8	76110	79967	105.07%
PM Liver 9	76110	81239	106.74%
PM Liver 10	76110	85208	111.95%
		Average	99.77%
		CV%	13.49%

	Mitragynine (399.2/226.1) Qual1		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Liver 1	46848	50103	106.95%
PM Liver 2	46848	49505	105.67%
PM Liver 3	46848	35759	76.33%
PM Liver 4	46848	41492	88.57%
PM Liver 5	46848	49235	105.10%
PM Liver 6	46848	48486	103.50%
PM Liver 7	46848	33309	71.10%
PM Liver 8	46848	46749	99.79%
PM Liver 9	46848	51071	109.02%
PM Liver 10	46848	52719	112.53%
		Average	97.86%
		CV%	13.85%

	Mitragynine (399.2/159.0) Qual2		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Liver 1	33276	36840	110.71%
PM Liver 2	33276	37907	113.92%
PM Liver 3	33276	25088	75.39%
PM Liver 4	33276	31657	95.13%
PM Liver 5	33276	35131	105.57%
PM Liver 6	33276	35273	106.00%
PM Liver 7	33276	23015	69.16%
PM Liver 8	33276	33455	100.54%
PM Liver 9	33276	35460	106.56%
PM Liver 10	33276	34747	104.42%
		Average	98.74%
		CV%	14.33%

Standard/Internal Standard Ratio				
	Standard-Quant	**ISTD-Quant**	**Resp Ratio**	**Deviation**
Neat (15 ng/mL)	76110	143408	0.5307	0.00%
PM Liver 1	84209	153915	0.5471	3.09%
PM Liver 2	82900	148229	0.5593	5.38%
PM Liver 3	60367	108218	0.5578	5.11%
PM Liver 4	73092	133881	0.5459	2.87%
PM Liver 5	81160	147504	0.5502	3.67%
PM Liver 6	78071	149441	0.5224	-1.56%
PM Liver 7	53141	102190	0.5200	-2.02%
PM Liver 8	79967	143616	0.5568	4.92%
PM Liver 9	81239	156717	0.5184	-2.33%
PM Liver 10	85208	152355	0.5593	5.38%
		Average	0.5437	2.45%

Table 13 Mitragynine (130 ng/0.1mL) Ion Suppression/Enhancement Data for Quant and Qualifier Ions for Liver

	Mitragynine (399.2/174.0) Quant		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Liver 1	770153	729629	94.74%
PM Liver 2	770153	685377	88.99%
PM Liver 3	770153	742376	96.39%
PM Liver 4	770153	754711	97.99%
PM Liver 5	770153	626680	81.37%
PM Liver 6	770153	716354	93.01%
PM Liver 7	770153	771241	100.14%
PM Liver 8	770153	777936	101.01%
PM Liver 9	770153	750149	97.40%
PM Liver 10	770153	715052	92.85%
		Average	94.39%
		CV%	5.86%

	Mitragynine (399.2/226.1) Qual1		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Liver 1	473595	451307	95.29%
PM Liver 2	473595	419647	88.61%
PM Liver 3	473595	451983	95.44%
PM Liver 4	473595	467018	98.61%
PM Liver 5	473595	381270	80.51%
PM Liver 6	473595	437897	92.46%
PM Liver 7	473595	473162	99.91%
PM Liver 8	473595	483124	102.01%
PM Liver 9	473595	456191	96.33%
PM Liver 10	473595	443291	93.60%
		Average	94.28%
		CV%	6.21%
	Mitragynine (399.2/159.0) Qual2		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Liver 1	347675	332387	95.60%
PM Liver 2	347675	307558	88.46%
PM Liver 3	347675	323326	93.00%
PM Liver 4	347675	335684	96.55%
PM Liver 5	347675	278303	80.05%
PM Liver 6	347675	319774	91.98%
PM Liver 7	347675	349169	100.43%
PM Liver 8	347675	347956	100.08%
PM Liver 9	347675	264219	76.00%
PM Liver 10	347675	327299	94.14%
		Average	91.63%
		CV%	8.35%

Standard/Internal Standard Ratio

	Standard-Quant	**ISTD-Quant**	**Resp Ratio**	**Deviation**
Neat (130 ng/mL)	770153	145436	5.2955	0.00%
PM Liver 1	729629	143201	5.0951	-3.78%
PM Liver 2	685377	144347	4.7481	-10.34%
PM Liver 3	742376	138487	5.3606	1.23%
PM Liver 4	754711	143601	5.2556	-0.75%
PM Liver 5	626680	120136	5.2164	-1.49%
PM Liver 6	716354	136340	5.2542	-0.78%
PM Liver 7	771241	148264	5.2018	-1.77%
PM Liver 8	777936	145043	5.3635	1.28%
PM Liver 9	750149	139473	5.3785	1.57%
PM Liver 10	715052	137979	5.1823	-2.14%
		Average	5.2056	-1.70%

Table 14 7-OH Mitragynine (10 ng/0.1mL) Ion Suppression/Enhancement Data for Quant and Qualifier Ions for Blood

	7-OH Mitragynine (415.2/190.1) Quant		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	14554	13344	91.68%
PM Blood 2	14554	10120	69.53%
PM Blood 3	14554	11577	79.54%
PM Blood 4	14554	11452	78.68%
PM Blood 5	14554	11291	77.58%
PM Blood 6	14554	12309	84.57%
PM Blood 7	14554	13166	90.46%
PM Blood 8	14554	10270	70.56%
PM Blood 9	14554	10971	75.38%
PM Blood 10	14554	13970	95.99%
		Average	81.40%
		CV%	10.50%

	7-OH Mitragynine (415.2/175.0) Qual1		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	5952	5516	92.68%
PM Blood 2	5952	3831	64.37%
PM Blood 3	5952	4662	78.33%
PM Blood 4	5952	4981	83.69%
PM Blood 5	5952	4748	79.78%
PM Blood 6	5952	5208	87.51%
PM Blood 7	5952	5296	88.99%
PM Blood 8	5952	4559	76.60%
PM Blood 9	5952	4575	76.87%
PM Blood 10	5952	5842	98.16%
		Average	82.70%
		CV%	11.06%

	7-OH Mitragynine (415.2/146.1) Qual2		
	Neat	**Post-Extraction**	**Matrix**
			0.00%
PM Blood 1	3868	3655	94.49%
PM Blood 2	3868	2634	68.10%
PM Blood 3	3868	3171	81.98%
PM Blood 4	3868	3228	83.45%
PM Blood 5	3868	3161	81.72%
PM Blood 6	3868	3184	82.32%
PM Blood 7	3868	3498	90.43%
PM Blood 8	3868	2968	76.73%
PM Blood 9	3868	2883	74.53%
PM Blood 10	3868	3708	95.86%
		Average	82.96%
		CV%	10.01%

Standard/Internal Standard Ratio				
	Standard-Quant	**ISTD-Quant**	**Resp Ratio**	**Deviation**
Neat (15 ng/0.1 mL)	14554	38329	0.3797	0.00%
PM Blood 1	13344	34721	0.3843	1.21%
PM Blood 2	10120	26872	0.3766	-0.82%
PM Blood 3	11577	31128	0.3719	-2.06%
PM Blood 4	11452	31114	0.3681	-3.07%
PM Blood 5	11291	30908	0.3653	-3.80%
PM Blood 6	12309	32463	0.3792	-0.15%
PM Blood 7	13166	35731	0.3685	-2.96%
PM Blood 8	10270	25493	0.4029	6.09%
PM Blood 9	10971	29486	0.3721	-2.01%
PM Blood 10	13970	36863	0.3790	-0.20%
		Average	0.3768	-0.78%

Table 15 7-OH Mitragynine (10 ng/0.1mL) Ion Suppression/Enhancement Data for Quant and Qualifier Ions for Liver

	7-OH Mitragynine (415.2/190.1) Quant		
	Neat	**Post-Extraction**	**Matrix**
PM Liver 1	14554	4200	28.86%
PM Liver 2	14554	1637	11.25%
PM Liver 3	14554	11293	77.59%
PM Liver 4	14554	2928	20.12%
PM Liver 5	14554	12903	88.65%
PM Liver 6	14554	12610	86.64%
PM Liver 7	14554	12719	87.39%
PM Liver 8	14554	11377	78.17%
PM Liver 9	14554	10259	70.49%
PM Liver 10	14554	12314	84.61%
		Average	63.38%
		CV%	45.89%

	7-OH Mitragynine (415.2/175.0) Qual1		
	Neat	**Post-Extraction**	**Matrix**
PM Liver 1	5952	1656	27.82%
PM Liver 2	5952	576	9.68%
PM Liver 3	5952	4809	80.80%
PM Liver 4	5952	1340	22.52%
PM Liver 5	5952	5175	86.95%
PM Liver 6	5952	5486	92.18%
PM Liver 7	5952	5097	85.64%
PM Liver 8	5952	4619	77.61%
PM Liver 9	5952	4196	70.50%
PM Liver 10	5952	5031	84.53%
		Average	63.82%
		CV%	46.22%

	7-OH Mitragynine (415.2/146.1) Qual2		
	Neat	**Post-Extraction**	**Matrix**
PM Liver 1	3868	1167	30.17%
PM Liver 2	3868	406	10.50%
PM Liver 3	3868	3176	82.11%
PM Liver 4	3868	830	21.46%
PM Liver 5	3868	3408	88.11%
PM Liver 6	3868	3490	90.23%
PM Liver 7	3868	3376	87.28%
PM Liver 8	3868	3167	81.88%
PM Liver 9	3868	2856	73.84%
PM Liver 10	3868	3388	87.59%
		Average	65.32%
		CV%	45.70%

Standard/Internal Standard Ratio

	Standard-Quant	**ISTD-Quant**	**Resp Ratio**	**Deviation**
Neat (15 ng/0.1 mL)	14554	38329	0.3797	0.00%
PM Liver 1	4200	10991	0.3821	0.63%
PM Liver 2	1637	3747	0.4369	15.05%
PM Liver 3	11293	31062	0.3636	-4.26%
PM Liver 4	2928	8123	0.3605	-5.07%
PM Liver 5	12903	34630	0.3726	-1.88%
PM Liver 6	12610	35064	0.3596	-5.29%
PM Liver 7	12719	36938	0.3443	-9.32%
PM Liver 8	11377	27978	0.4066	7.09%
PM Liver 9	10259	28260	0.3630	-4.40%
PM Liver 10	12314	32734	0.3762	-0.93%
		Average	0.3765	-0.84%

Carryover

Carryover was assessed by fortifying blood and liver homogenate with 800 ng/0.1 mL of mitragynine and 500 ng/0.1 mL of 7-OH then analyzing a blank matrix sample immediately following the high concentration sample. There was no carryover present in any of the blank samples for blood or liver, evidenced by no analyte signal following the high concentration samples.

Limit of Detection

The LOD for mitragynine was established at 1.0 ng/0.1 mL. This was evaluated over three different calibration curves with each LOD sample run in duplicate in three different blank matrix samples (blood and liver homogenate). To establish LOD, the integration of one parent and one daughter ion, good discernable peak shape, retention times had to match the times in the calibration curve, and a signal to noise ratio of 3:1 is needed. 7-OH LOD was administratively set at 10 ng/0.1mL. This was evaluated over three different calibration curves with each 10 ng calibrator run in three different blank matrix samples (blood and liver homogenate). Figures 6 and 7 depict the LC/MS-MS chromatographic data for the LOD samples for mitragynine and 7-OH respectively.

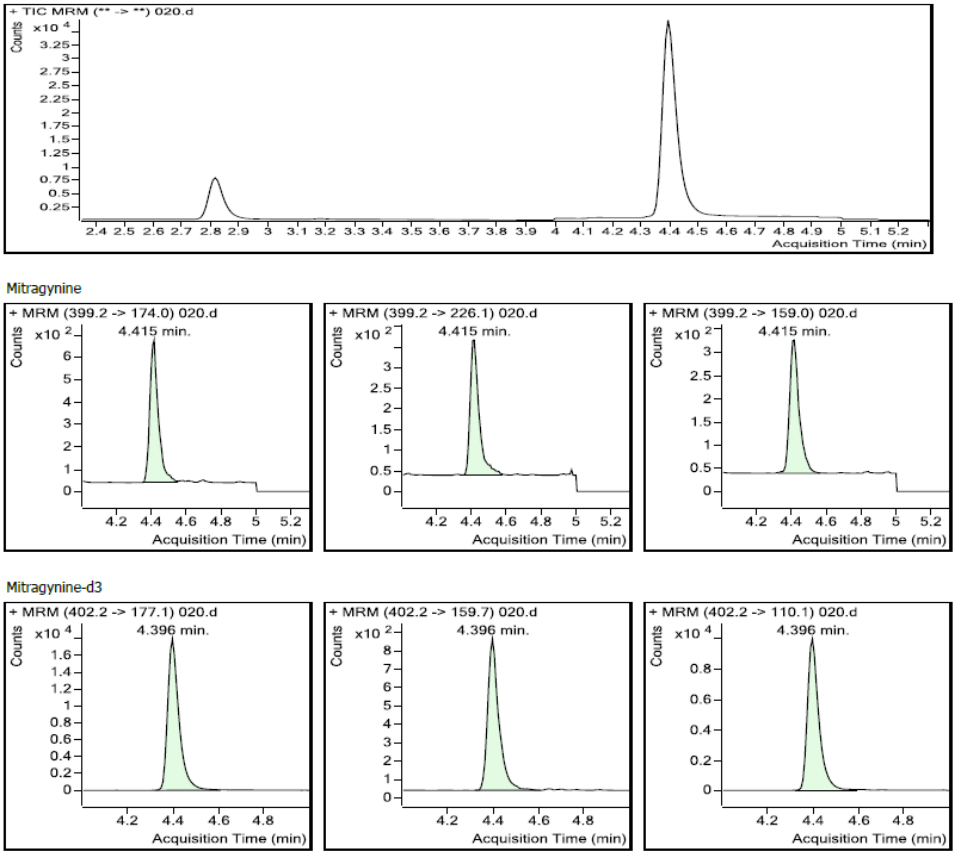
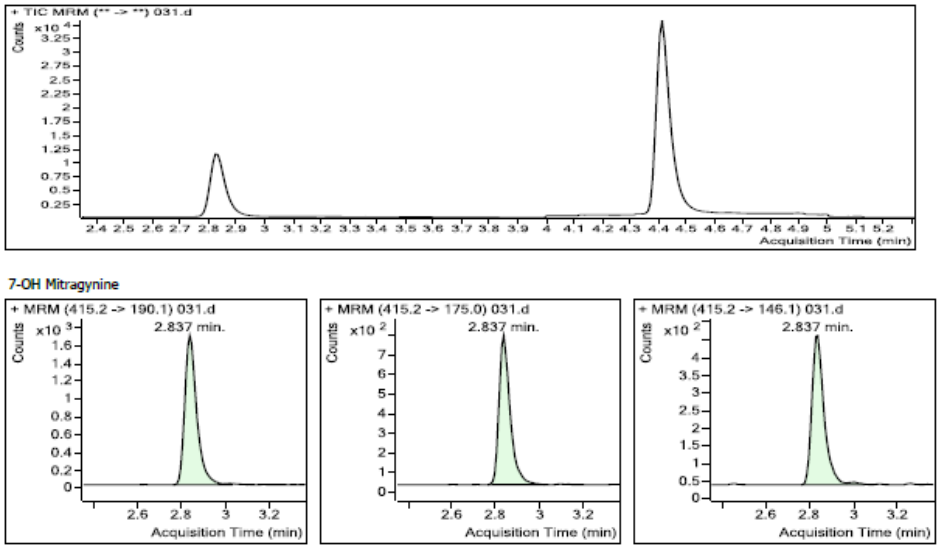


Figure 6. Chromatogram of mitragynine at LOD concentration of 1 ng/0.1 mL.



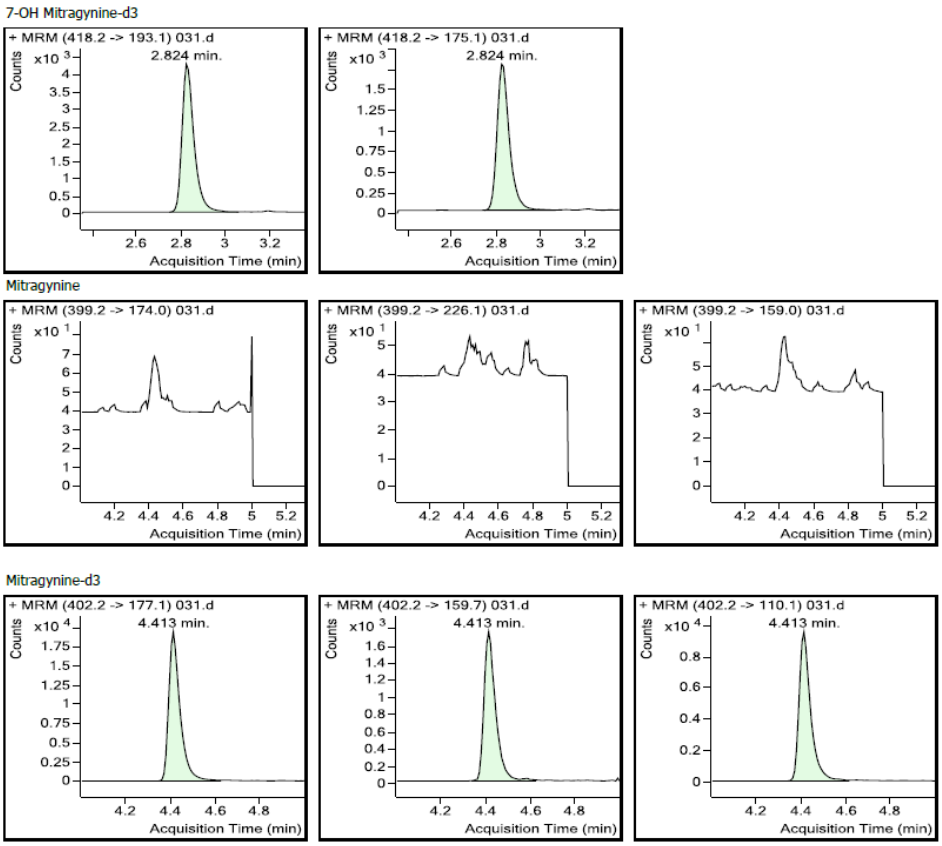


Figure 7. Chromatogram of 7-OH at LOD concentration of 10 ng/0.1 mL.

Calibration Model

The calibrators chosen for this method validation were 5, 10, 20, 40, 80, and 160 ng/0.1 mL. Results were assessed against multiple regression models with various weighting factors. Due to the linear range extending beyond one order of magnitude, a 1/x weighted linear model was selected and applied. In addition to the calibrators, a negative control (internal standard only) and a blank (no drug or internal standard) were prepared in the laboratory's blank blood matrix (control blood). The blank was injected after the highest calibrator to ensure there was no carryover from the calibration curve. The negative control, calibrators, blank, and quality control samples (run at three different levels in triplicate) were analyzed over five separate runs. Table 16 shows trials 1-5 calibrator concentrations, drug response, internal standard response, and area

ratio. The concentration versus area ratio was plotted into a line graph for each trial with the 1/x weight shown in Figure 8. Figure 9 plots the concentration (1/x) versus the standard residuals for all five trials.

Table 16 Calibration Model Data

Trial 1:

Calibrator Level & Concentration	ng/ 0.1 mL	Drug Response	Internal Standard Response	Area Ratio
L1	5	13755	75057	0.183
L2	10	31211	79685	0.392
L3	20	67799	73580	0.921
L4	40	159211	87000	1.830
L5	80	311482	76213	4.087
L6	160	724444	95175	7.612

Trial 2:

Calibrator Level & Concentration	ng/ 0.1 mL	Drug Response	Internal Standard Response	Area Ratio
L1	5	15970	84185	0.190
L2	10	16353	43343	0.377
L3	20	28274	33391	0.847
L4	40	58349	31580	1.848
L5	80	284485	71715	3.967
L6	160	620402	93931	6.605

Trial 3:

Calibrator Level & Concentration	ng/ 0.1 mL	Drug Response	Internal Standard Response	Area Ratio
L1	5	18340	115151	0.159
L2	10	42115	124017	0.340
L3	20	93813	118911	0.789
L4	40	148636	84215	1.765
L5	80	374321	105034	3.564
L6	160	702817	102145	6.881

Trial 4:

Calibrator Level & Concentration	ng/ 0.1 mL	Drug Response	Internal Standard Response	Area Ratio
L1	5	18699	112819	0.166
L2	10	38594	106560	0.362
L3	20	90138	110730	0.814
L4	40	168086	98892	1.700
L5	80	377619	105766	3.570
L6	160	737161	101146	7.288

Trial 5:

Calibrator Level & Concentration	ng/ 0.1 mL	Drug Response	Internal Standard Response	Area Ratio
L1	5	18557	87341	0.212
L2	10	49459	112160	0.441
L3	20	90564	90847	0.997
L4	40	206971	96672	2.141
L5	80	466226	111708	4.174
L6	160	700794	78405	8.938

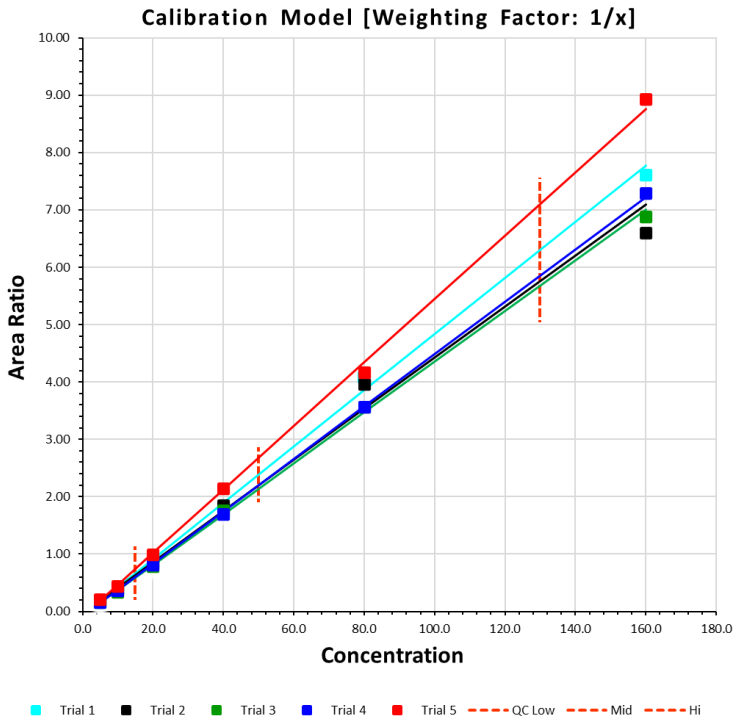


Figure 8. Concentration versus area ratio for trial 1-5 used for the calibration model.

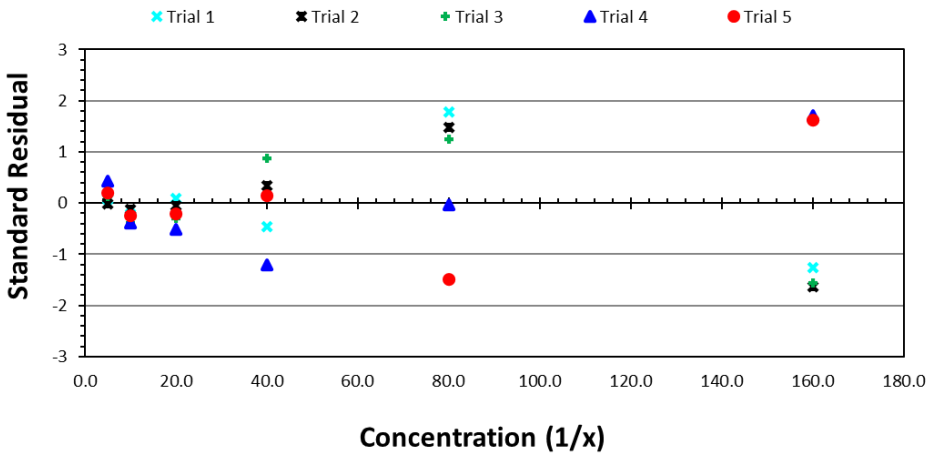


Figure 9. Concentration versus standard residuals for trials 1-5 used for the assessment of the calibration model.

Stability and Degradation Results

Autosampler stability was evaluated by extracting a low (15ng/0.1mL) and high (130ng/0.1mL) mitragynine QC sample in blood and liver and a 7-OH (10ng/0.1mL) QC sample, injected them immediately (T_0) and leaving them on the cooled autosampler. Samples were re-injected 24, 48, and 72 hour post-extraction. All results were compared to T_0 results to determine the analyte's stability as a processed sample. Area response of the processed samples did not decrease over the time intervals tested and all QC samples were in range and properly identified. Processed samples are considered stable up to 72 hours.

Long-term stability was evaluated at low and high concentrations for mitragynine and at a mid-level concentration for 7-OH in triplicate, in three different storage conditions. Ambient conditions were evaluated at 0, 24, 48, and 72 hours, while refrigerated and frozen samples were evaluated at 7, 14, 21, 30, 65, and 90 days.

Ambient

The low concentration of mitragynine decreased 5% over a 72-hour. The high concentration of mitragynine did not have a depreciable change over 72 hours. 7-OH mitragynine decreased

15% over the 72-hour period. Figure 10 depicts the low and high concentrations assessed for mitragynine and 7-OH at ambient temperature.

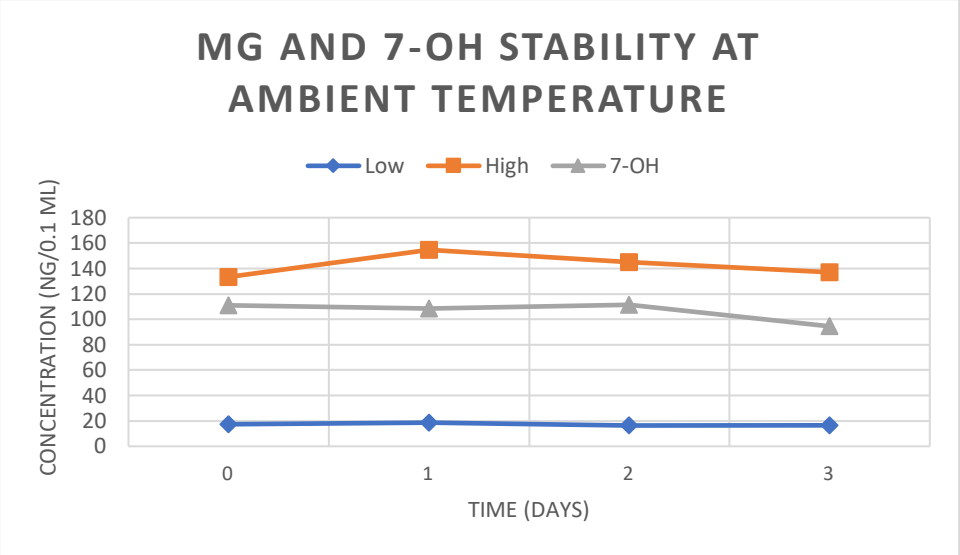


Figure 10. Time (hours) versus concentration (ng/0.1mL) for low and high concentrations of mitragynine and 7-OH at ambient temperature.

Refrigerated

The low concentration of mitragynine decreased 11% over a 30-day period. The high concentration did not have a depreciable change over 30 days. 7-OH mitragynine decreased 10% over a 30-day period. The low concentration of mitragynine decreased by 17% after 65 days in refrigerator temperatures. The high concentration did not decrease over the 65 or 90 days. The 7-OH decreased by 27% after 65 days. After the full 90 days, the low concentration decreased by 25% and the 7-OH did not decrease from 65 days to 90 days. Figure 11 depicts the days versus concentration for low and high concentrations of mitragynine and 7-OH at refrigerated (4^{o}C) temperature.

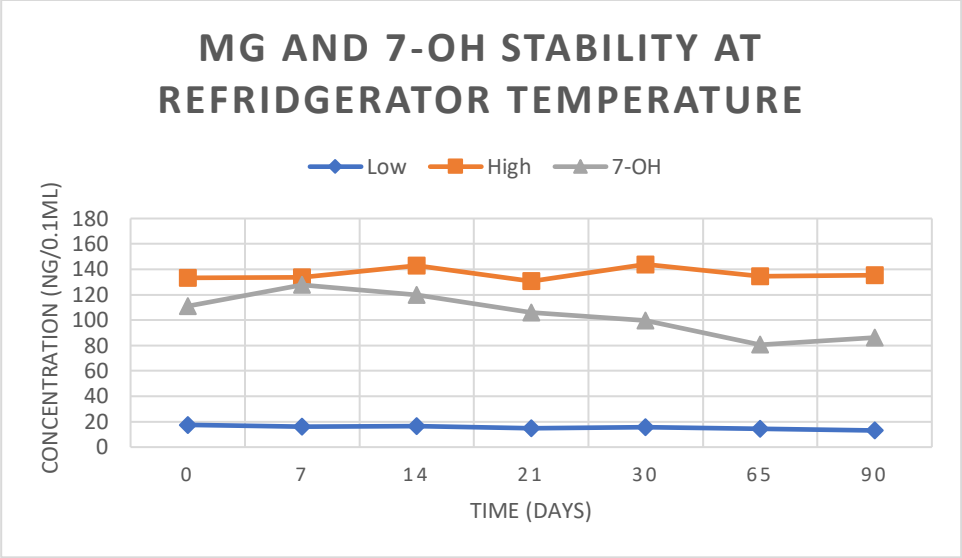


Figure 11. Time (Days) versus concentration (ng/0.1mL) for low and high concentrations of mitragynine and 7-OH at refrigerator temperature.

Frozen

The low concentration of mitragynine decreased 73% over a 30-day period. The high concentration decreased 54% over 30 days. 7-OH decreased 6% over a 30-day period. The low concentration decreased by 79% by 65 days and did not have a depreciable change by 90 days. The high concentration decreased by 65% at 65 days and did not have a decrease at 90 days. The 7-hydroxymitragynine decreased by 9.5% over 65 days and did not have more degradation by 90 days. Figure 12 shows the days versus concentration of low and high concentrations of mitragynine and 7-OH at freezer (-30°C) temperature.

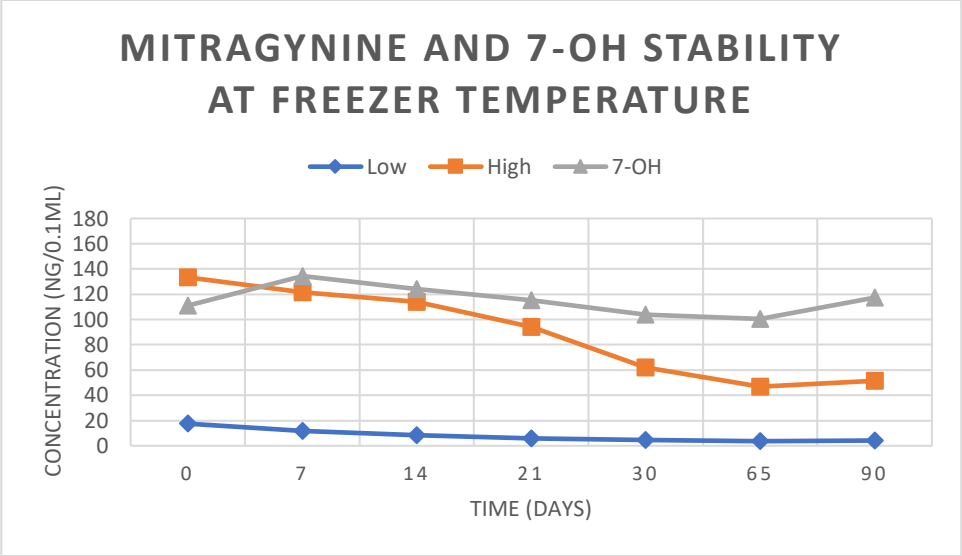


Figure 12. Time (Days) versus concentration (ng/0.1mL) for low and high concentrations of mitragynine and 7-OH at freezer temperature.

Case Studies

Case Study 1

The decedent was a 29-year-old white male living in a sober living home. The decedent's roommates went to check on him and he was found unresponsive. The police department was alerted and then responded to the scene and pronounced him dead. The decedent had a history of drug usage including fentanyl. Drug paraphernalia was found on the scene including multiple pieces of foil, a straw, and two lighters.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was negative for ethyl alcohol and the immunoassay came back positive for fentanyl. The alkaline drug screen identified the following drugs: para-fluorofentanyl, mitragynine, and fentanyl. The para-fluorofentanyl was confirmed in the femoral blood by a fentanyl analogue screen. The fentanyl was quantitated in the femoral blood and the

concentration was 11 ng/mL. The mitragynine was quantitated and the 7-hydroxymitragynine was qualitatively identified. The mitragynine concentration was 570 ng/mL in the femoral blood and 510 ng/g in the liver. The cause of death was determined as combined drug toxicity due to fentanyl, para-fluorofentanyl, and mitragynine. The manner of death was deemed as an accident.

Case Study 2

The deceased was a 28-year-old white male found unresponsive in a parked vehicle. 911 was called and death was pronounced on the scene. The decedent was known to have chronic poly-substance abuse. Drug paraphernalia including bags of "Kratom" were found on scene.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was negative for ethyl alcohol and the immunoassay came back positive for fentanyl. The alkaline drug screen identified fentanyl and mitragynine. The fentanyl was quantitated in the femoral blood and had a concentration of 32 ng/mL. The mitragynine was quantitated and 7-hydroxymitragynine was qualitatively identified. The concentration of mitragynine in the heart blood was 400 ng/mL, 330 ng/mL in the femoral blood and 1,100 ng/g in the liver. The cause of death was determined to be combined drug toxicity due to fentanyl and mitragynine. The manner of death was deemed an accident.

Case Study 3

The deceased was a 34-year-old white male found unresponsive in their living room. 911 was called and cardiopulmonary resuscitation (CPR) was started. Two doses of naloxone were given by police on the scene. No drug paraphernalia was noted on the scene. The decedent did have a history of methamphetamine abuse. Their prescription medications found were all in count.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was negative for ethyl alcohol and the immunoassay came back negative. Methadone was identified on the alkaline drug screen and was then quantitated. The concentration of methadone was 0.48 μg/mL in the femoral blood. Sertraline was identified on the alkaline drug screen and later quantitated using femoral blood. The concentration of sertraline was 0.34 μg/mL. Gabapentin was first screened and then quantitated in the femoral blood and the concentration was 11 μg/mL. The mitragynine was quantitated and 7-hydroxymitragynine was qualitatively identified. The concentration of mitragynine in the heart blood was 440 ng/mL, 490 ng/mL in the femoral blood and 1,100 ng/g in the liver. The cause of death was determined to be a methadone toxicity. Condition contributing to death was atherosclerotic cardiovascular disease. The manner of death was deemed an accident.

Case Study 4

The deceased was a 32-year-old white male found unresponsive in his residence by his roommate. Emergency Medical Services (EMS) pronounced the decedent dead on the scene and suspected an overdose. Drug paraphernalia was found on the scene including a glass pipe and straw. Naloxone and prescription pills were also found in the room.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was negative for ethyl alcohol and the immunoassay came back positive for benzoylecgonine and fentanyl. The benzoylecgonine was quantitated in the femoral blood but was less than 0.05 μg/mL. The fentanyl was quantitated, and the concentration was 17 ng/mL in the femoral blood. The mitragynine was quantitated and 7-hydroxymitragynine was

qualitatively identified. The concentration of mitragynine in the heart blood was 150 ng/mL, 150 ng/mL in the femoral blood and 520 ng/g in the liver. The cause of death was deemed an acute combined drug (fentanyl, mitragynine, cocaine) toxicity. The condition contributing to death was hypertensive atherosclerotic cardiovascular disease. The manner of death was determined to be an accident.

Case Study 5

The deceased was a 30-year-old white female found by a neighbor in her residence. They were pronounced dead when found by 911. The decedent was in the beginning stages of decomposition. The decedent was also known to use heroin.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was not tested for ethyl alcohol because of the decomposition and the immunoassay came back positive for methamphetamine and fentanyl. The fentanyl was quantitated, and the concentration was 30 ng/g in the femoral blood. The methamphetamine was quantitated using femoral blood and the concentration was found to be 0.11 μg/mL. Pramoxine was detected by alkaline drug screen but was not confirmed. The mitragynine was quantitated and 7-hydroxymitragynine was qualitatively identified. The concentration of mitragynine in the femoral blood was 150 ng/mL and 580 ng/g in the liver. The cause of death was fentanyl and methamphetamine toxicity. The manner of death was deemed an accident.

Case Study 6

The deceased was a 54-year-old white female with possible suicidal ideations. The decedent was found unresponsive by their family and 911 was called. The decedent was transferred to the

emergency room and then pronounced shortly after arrival. Prescription medicine including three bottles of Zoloft and one bottle of clonidine were found empty.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was positive for ethyl alcohol (0.13 g/dL) and the immunoassay was negative. Several drugs including sertraline, doxepin, nordoxepin, and clonidine were all identified. The concentrations were 0.68 μg/mL (sertraline), 6.2 μg/mL (doxepin), <0.50 μg/mL (nordoxepin) and positive (clonidine). All of the quantitative analyses were performed on a hospital blood sample collected in the emergency room. The mitragynine was quantitated and 7-hydroxymitragynine was qualitatively identified. The concentration of mitragynine in the heart and femoral blood was 130 ng/mL, and 220 ng/g in the liver. The cause of death was determined to be doxepin, sertraline, clonidine and ethanol toxicity. The manner of death was suicide.

Case Study 7

The deceased was a 27-year-old white male found in the garage of his residence. EMS responded and death was pronounced. The decedent died of a suspected overdose. He was recently in a facility for his history of opiate abuse.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was negative for ethyl alcohol and the immunoassay was positive for fentanyl. Diphenhydramine was reported as positive in the case, and despropionyl fentanyl and naloxone were reported in trace amounts. Fentanyl was quantitated and the concentration in the femoral blood was 52 ng/mL. The mitragynine was quantitated and 7-hydroxymitragynine was qualitatively identified. The concentration of mitragynine in the heart blood was 540 ng/mL, 570

ng/mL in the femoral blood and 490 ng/g in the liver. The cause of death was determined to be acute combined drug toxicity due to fentanyl and mitragynine. Conditions contributing to death were hypertensive cardiovascular disease and obesity. The manner of death was deemed as an accident.

Case Study 8

The deceased was a 25-year-old white female found in their home and pronounced dead by EMS. The husband started CPR upon finding the decedent and also administered naloxone. The family denied any drug history. The prescription of gabapentin was short. The decedent had told their family that they had trouble breathing leading up to their death.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was negative for ethyl alcohol and the immunoassay was negative. Gabapentin was screened for due to the decedent's history but was negative. Oxycodone was identified and then quantitated at 0.29 μg/mL. Venlafaxine was also identified and quantitated at 0.85 μg/mL. The quantitative analyses were completed using femoral blood. The mitragynine was quantitated and 7-hydroxymitragynine was qualitatively identified. The concentration of mitragynine in the heart and femoral blood was 420 ng/mL and 680 ng/g in the liver. The cause of death was combined drug (oxycodone, venlafaxine, mitragynine) toxicity. The manner of death was determined to be an accident.

Case Study 9

The deceased was a 42-year-old white male found unresponsive in their bed by family. 911 was called and the fire department arrived and pronounced them dead. Pink pills were found in the deceased pocket. The decedent had a history of prescription drug abuse.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was negative for ethyl alcohol and the immunoassay was negative. 7-aminoclonazepam was identified and quantitated, and it was found to be 200 ng/mL in the femoral blood. Pregabalin was identified and quantitated using the femoral blood and the concentration was 30 μg/mL. The mitragynine was quantitated and 7-hydroxymitragynine was qualitatively identified. The concentration of mitragynine in the heart blood was 810 ng/mL, 710 ng/mL in the femoral blood and 2,300 ng/g in the liver. 7-OH was positively identified. The cause of death was determined to be combined drug toxicity (clonazepam, pregabalin, mitragynine). The manner of death was deemed an accident.

Case Study 10

The deceased was a 44-year-old Hispanic male was witnessed to take a Viagra and then get dizzy. After becoming dizzy, the decedent became unresponsive. 911 was called and the deceased was transported to the ER. They were pronounced as dead shortly after arrival.

Postmortem examination consisted of an autopsy examination and toxicology testing. During the toxicological testing, ethyl alcohol, immunoassay, and an alkaline drug screen were utilized. The decedent was negative for ethyl alcohol and the immunoassay was negative. The mitragynine was quantitated and 7-hydroxymitragynine was qualitatively identified. The concentration of mitragynine in the heart blood was 1,400 ng/mL, 1,300 ng/mL in the femoral blood and 7,000 ng/g in the liver. The presence of 7-OH was positively identified. The cause of death was mitragynine intoxication complicating hypertensive and arteriosclerotic cardiovascular disease. The manner of death was determined to be an accident.

Table 17 Mitragynine Case Values

Mitragynine Case Values							
Case Number	Heart Blood (ng/mL)	Femoral Blood (ng/mL)	Liver (ng/g)	7-OH	C/P	L/P	
Case 1	N/A	570	510	Negative	N/A	0.89	
Case 2	400	330	1,100	Negative	1.2	3.3	
Case 3	440	490	1,100	Negative	0.90	2.2	
Case 4	150	150	520	Negative	1.0	3.5	
Case 5	N/A	150	580	Negative	N/A	3.7	
Case 6	130	130	220	Negative	1.0	1.7	
Case 7	540	570	490	Negative	0.95	0.86	
Case 8	420	420	680	Negative	1.0	1.6	
Case 9	810	710	2,300	Positive	1.1	3.2	
Case 10	1,400	1,300	7,000	Positive	1.1	5.4	
Range	130-1,400	130-1,300	220-7,000		1.03	2.7	Average
					0.10	1.4	Standard deviation

Table 18 Case Study Information

Case	*Age*	*Sex*	*Race*	*Scene Information*	*Drug Paraphernalia*	*COD*	*MOD*	*Heart Blood (ng/mL)*	*Femoral blood (ng/mL)*	*Liver (ng/g)*	*7-OH*	*Additional toxicological findings*
1	29	M	White	Found unresponsive by roommate. Police alerted to scene and pronounced dead. History of fentanyl use.	Y	Combined drug toxicity due to fentanyl, para-fluorofentanyl and mitragynine	Accident	N/A	570	510	N/A	Fentanyl: 11 ng/mL Para-fluorofentanyl: Positive
2	28	M	White	Found unresponsive in a parked vehicle. 911 called and death pronounced at scene. Chronic poly-substance abuse.	Y	Combined drug toxicity due to fentanyl and mitragynine	Accident	400	330	1,100	N/A	Fentanyl: 32 ng/mL
3	34	M	White	Found unresponsive in living room. 911 was called and CPR started. Administered two doses naloxone. History of methamphetamine abuse.	N	Methadone toxicity	Accident	440	490	1,100	N/A	Methadone: 0.48 µg/mL Sertraline: 0.34 µg/mL Gabapentin: 11 µg/mL
4	32	M	White	Found unresponsive in residence by roommate. EMS pronounced decedent dead.	Y	Acute combined drug (fentanyl, mitragynine, cocaine) toxicity	Accident	150	150	520	N/A	Benzoylecgonine: < 0.05 µg/mL Fentanyl: 17 ng/mL
5	30	F	White	Found by neighbor in residence. 911 called and pronounced dead. Known to use heroin.	N	Fentanyl and methamphetamine toxicity	Accident	N/A	150	580	N/A	Fentanyl: 30 ng/g Methamphetamine: 0.11 µg/mL Pramoxine: Detected not confirmed

Table 18 continued.

Case	*Age*	*Sex*	*Race*	*Scene Information*	*Drug Paraphernalia*	*COD*	*MOD*	*Heart Blood (ng/mL)*	*Femoral blood (ng/mL)*	*Liver (ng/g)*	*7-OH*	*Additional toxicological findings*
6	54	F	White	Found unresponsive by family. Transferred to ER and pronounced shortly after arrival.	Y	Doxepin, sertraline, clonidine, and ethanol toxicity	Suicide	130	130	220	N/A	Ethyl alcohol: 0.13 g/dL Sertraline: 0.68 µg/mL Doxepin: 6.2 µg/mL Nordoxepin: <0.5 µg/mL Clonidine: Positive
7	27	M	White	Found unresponsive in garage. He was in facility for history of opiate abuse.	N	Acute combined drug toxicity due to fentanyl and mitragynine	Accident	540	570	490	N/A	Fentanyl: 52 ng/mL Diphenhydramine: positive Despropionyl fentanyl: trace Naloxone: trace
8	25	F	White	Found unresponsive in home. Pronounced dead by EMS. CPR was started and naloxone was administered before death.	N	Combined drug (oxycodone, venlafaxine, mitragynine) toxicity	Accident	420	420	680	N/A	Oxycodone: 0.29 µg/mL Venlafaxine: 0.85 µg/mL
9	42	M	White	Found unresponsive in bed by family. Fire department arrived and pronounced them dead.	Y	Combined drug toxicity (clonazepam, pregabalin, mitragynine)	Accident	810	710	2,300	Positive	7-aminoclonazepam: 200 ng/mL Pregabalin: 30 µg/mL
10	44	M	Hispanic	Witnessed to take a Viagra then get dizzy. Decedent became unresponsive and then was transported to the ER where he was pronounced shortly after arrival.	N	Mitragynine intoxication complicating hypertensive and arteriosclerotic cardiovascular disease	Accident	1,400	1,300	7,000	Positive	N/A

Discussion

Mitragynine was chosen as the analyte of choice for the method validation, stability study, and case studies because it is the most abundant alkaloid in kratom. A rise in mitragynine cases identified at the OCME also made mitragynine a good choice for the study. 7-OH was qualitatively identified, the stability was monitored and identified in case studies because it is more psychoactive than mitragynine. 7-OH was not quantitated because it has known stability issues which were confirmed by our stability study and is not found to be abundant in the plant material. Cases containing mitragynine were rising at the OCME for years prior to this project, highlighting the need for a validated method.

The project led to a validated method for the quantitation of mitragynine and the qualitative identification of 7-OH. The stability study generated data for the degeneration of mitragynine and 7-OH in blood in ambient, refrigerated, and frozen conditions. Case studies were completed using real world cases received by OCME Toxicology Laboratory. The data from the case studies can provide estimates on the range of mitragynine concentrations in postmortem heart blood, femoral blood, and liver. These concentrations were also used to calculate the central blood to peripheral blood (C/P) and liver to peripheral blood (L/P) ratios. These ratios can aid in determining if mitragynine is prone to postmortem redistribution.

The extraction used for the method was evaluated in the method development stage. A forward alkaline extraction including the use of ammonium hydroxide and butyl chloride was first explored. The alkaline extraction was not a good fit for the analytes because of the high pH change, resulting in the degradation of mitragynine into 7-OH. An acetonitrile crash was also tested in the method development stage, and the method did not convert mitragynine to the same magnitude but there was low recovery of mitragynine. The SLE method used in the study was

the best choice because the conversion of mitragynine was minimal and it produced good recovery of analytes.

In the literature, other extractions utilized included liquid-liquid extraction (LLE) and solid phase extraction (SPE), but none of the other methods employed a SLE method (Kerrigan & Basiliere, 2022). The SLE method uses solvents in the extraction, but does not take as long as a LLE extraction. The SLE is less expensive because the SLE only utilizes a diatomaceous earth column instead of solid phase cartridges. Inert material (diatomaceous earth) is used for SLE and the entire sample is poured onto the column and the analytes are then selectively eluted using an organic solvent, leaving the matrix behind on the column. On the other hand, when using SPE the matrix and interferences pass through the sorbent material to waste.

The method validation assessed interferences, bias, precision, matrix effects, limit of detection, carryover, calibration model, and autosampler stability for mitragynine. 7-OH was evaluated for interferences, matrix effects, limit of detection, carryover and autosampler stability. The parameters evaluated in the method validation were based on ASB recommendations for quantitative and qualitative methods.

Interference testing was used to identify endogenous matrix interferences and interferences from commonly seen compounds. The endogenous matrix interference study used blank matrix (blood and liver) samples from ten different sources without the addition of internal standard. This demonstrates that there are no interferences from the endogenous matrix which could interfere with the analyte of interest. A quality control sample at a low concentration with internal standard, a 7-OH test mix, and a blank matrix sample were extracted in control blood to compare potential matrix artifacts in postmortem samples.

Interferences from commonly seen compounds utilized five multi-drug mixes which contained drugs which are commonly seen in the OCME laboratory. The mixes were extracted in ten sources of blood and liver samples. A negative control with internal standard and high concentration quality control with no internal standard, and a 7-OH test mix were analyzed. No interferences were detected for any of the drugs in the mixes.

Bias was evaluated in conjunction with precision and needs to have a coefficient of variation less than 20%. The bias requirement was met by mitragynine and was no higher than 8% in both the liver and blood samples. The precision was also evaluated and needed to be less than 20% for blood and liver trials. The precision was calculated for within-run and between-run. The within-run precision measures the concentration separately for each of five runs. The between-run precision is calculated from the combined data from all replicates of each level of concentration. The precision was no greater than 9% for between run and within run precision for liver and blood studies.

The matrix effects or ion suppression/enhancement were completed in this study since the method utilized LC/MS-MS analysis. A matrix effect can occur when a closely eluted compound either suppresses or enhances the ionization of the analyte molecules in the mass spectrometer's source. A quality control sample of the assay and the internal standard or a "neat" sample is compared to a sample which undergoes extraction. Ten replicated of each were compared to assess the percentage of suppression or enhancement.

All ion suppression/enhancement for mitragynine and 7-OH was within the required limits from the OCME. The liver samples for 7-OH showed large ion suppression but the standard to internal standard ratios averaged less than 15% deviation, meaning that the standard and internal standard were suppressed similarly. Also, 7-OH was only qualitatively identified for the project

so there were no criteria placed on allowable matrix effect. The mitragynine blood samples also had some ion suppression, but the standard to internal standard ratio average was below 15% deviation. There were several bloods which had worse suppression including postmortem blood number 10 seen in Table 11.

The mitragynine high QC for a single transition attained a %CV at 15% due solely to an outlier associated with PM Blood 10. This sample was heavily decomposed and demonstrated comparable suppression for all transitions in both the drug and internal standard; however, standard/internal standard ratios were within limits. Heavily decomposed specimens show some ion suppression and/or enhancement for individual blood and liver in both the low and high control samples. The internal standard was affected equally, therefore the ability to accurately quantitate the analyte should not be impacted. In dealing with the potential for poor quality specimens during routine analysis, an additional tissue sample fortified with low QC material will be extracted to check for matrix effect on a case-by-case basis. In the case of an unknown blood sample showing internal standard recovery outside 50-200% of a control internal standard recovery, an additional sample fortified with low QC sample will be re-extracted with the case to check for matrix effect.

Carryover was evaluated for mitragynine and 7-OH. A sample at 1.0 ng/0.1 mL for mitragynine and 10 ng/0.1mL for 7-OH was extracted and injected in triplicate to get an average signal. A sample which was over the expected concentration for mitragynine (800 ng/0.1mL) and 7-OH (500 ng/0.1mL) was injected followed by a blank blood sample that underwent extraction. This was repeated several times and was assessed in blood and liver. There was no carryover in any of the blank samples for blood or liver. In the future, if there was suspected carryover in a case then the case would be repeated.

Limit of detection was evaluated for mitragynine and 7-OH and represents the lowest analyte concentration for which a specific identification criteria can be fulfilled. The LOD for mitragynine was established at 1.0 ng/0.1mL. Three calibration curves with each LOD sample run in duplicate in three different blank matrix samples (liver and blood) were used to assess the LOD. The signal to noise ratio was greater than 3:1, there was good discernable peak shape, and at least one set of ion ratios were within 20% for each sample, and retention times matched the calibration curve standards. Throughout the calibration range for mitragynine, a conversion of mitragynine to 7-OH was observed and increased with the concentration. The conversion from mitragynine to 7-OH was less than 1% during the extraction process, but a discernable peak was present and integrating at an approximate concentration of 1 ng/0.1mL. Therefore, the LOD for 7-OH was administratively set at a decision point of 10 ng/0.1mL. That concentration was evaluated over three different calibration curves with each 10 ng calibrator run in three different blank matrix samples for blood and liver. All identification criteria were met.

The calibration model was evaluated using calibrators at 5, 10, 20, 40, 80, and 160 ng/0.1mL. A calibrator at 2.5 ng/0.1mL was assessed in method development but was dropped for the validation to have a six-point curve which evaluates up to 160 ng/0.1mL. The weighted linear model 1/x was selected and applied since the linear range extended beyond one order of magnitude. Figure 8 depicted the concentration versus area ratio which had more variation between runs at higher concentrations, which is the reason for the 1/x calibration model.

In the autosampler stability study, the area response of the processed samples did not decrease over the four-time intervals tested and all QCs were in range and properly identified. Processed samples are considered stable up to 72 hours.

In the stability study, the refrigerated samples of mitragynine paradoxically appear to demonstrate greater stability when compared to frozen samples; however, it is encouraged to identify and quantitate mitragynine in specimens in a timely manner. Papsun et al also did a 90-day stability study and found that mitragynine was stable at all temperatures up to 30 days. From 30-90 days mitragynine had a 20% decrease at all temperatures. They also stated that a longer period between specimen collection and testing would decrease the accuracy of the results. Parhasarathy did three freeze thaw cycles and found mitragynine to be stable in plasma.

Papsun's results compare to this study in the level of degradation mitragynine had for all temperatures. On the other hand, this study found that frozen temperatures had more effect on the degradation than refrigerator temperatures. Papsun evaluated frozen temperatures at both -70℃ and -25℃ and while they both degraded by 20% between 30-90 days, the degradation for the refrigerated temperature was worse. It can be assumed that the lower temperature is not degrading the mitragynine to a higher degree because of Papsun's results, so the thawing of the specimens in the specific matrix could be further assessed.

On the other hand, 7-OH demonstrated greater stability in frozen conditions than the refrigerated. No other studies have assessed the stability of 7-OH in a blood matrix, so there are no comparisons to make for this drug. When purchasing a reference standard for 7-OH the manufacturers suggest that the methanolic sample is stored at subfreezing temperatures because of its known instability. The difference between stability for mitragynine and 7-OH poses an issue for laboratory testing. Since mitragynine has shown to be more stable at refrigerated temperatures and 7-OH at frozen temperatures, then one drug will inevitably be favored by storage conditions, and the other will degrade more quickly. Since mitragynine is being

quantitated by the OCME laboratory then the refrigerated temperatures will be more favorable. Samples stored for extended periods of time should be interpreted with caution.

In the case studies, the demographics of decedents was 60% white males. 30% of the cases were white females, and one of the cases was a Hispanic male. The age range of the decedents was 25-54 years, with the average age being 35 years old. Nine of the decedents had drugs other than mitragynine in their system upon death, and seven of the cases cited mitragynine in their cause of death. Of the cases with a cause of death determined, the manner of death was accident except for one of the cases which was ruled a suicide.

Papsun et al mention that between 2014 to 2017 there were 6 driving under the influence of drugs (DUID) cases involving mitragynine. Then in 2019 there were 20 DUID cases reported with a concentration range of 11-490 ng/mL. This range is important since there are no reported clinical trials for mitragynine, therefore; the concentration for impairment or even toxicity is unknown. The DUID mitragynine concentration range gives insight into the concentration of mitragynine that may cause psychomotor impairment, giving toxicologists more tools to interpret a case.

The range of postmortem concentrations in this project for heart blood was 130-1,400 ng/mL and the range for femoral blood was 130-1,300 ng/mL. The range of concentrations for liver was 220-7,000 ng/g. The average concentration for heart blood was 536 ng/mL, 482 ng/ mL for femoral blood and 1,450 ng/g for liver. The median concentration for heart and femoral blood was 420 ng/mL and 580 ng/g for liver. Prior reports in the literature describe a mean of 410 ng/mL (Papsun et al., 2019) and 625 ng/mL (heart blood), 903 ng/mL (femoral blood) (Mata & Andera, 2020). Mata & Andera's averages were both higher than those found in this study but that could be associated with the higher number of cases assessed. The median reported by

Papsun et al was 130 ng/mL which was lower than our median, while their range was much higher (5.6-29,000 ng/mL). Only two out of the ten cases performed in the current study had a positive 7-OH indicating they were larger than the administrative LOD set at 10 ng/0.1mL. 7-OH was also identified in Mata & Andera's paper in two of their ten screened cases.

The C/P ratio ranged from 0.90-1.14 and had an average of 1.03. The L/P ratio ranged from 0.86-5.38 and had an average of 2.66. Both the central to peripheral and liver to peripheral ratios suggest that mitragynine does not undergo postmortem redistribution, which agrees with the prior studies found in the literature (Mata & Andera, 2020; I. M. McIntyre et al., 2015). Mata & Andera had C/P ratios between 0.04-1.26 which is a large range compared to this study's ratios which were fairly consistent. Many of Mata and Andera C/P ratios suggested that femoral blood concentrations were typically higher than heart blood, which is not consistent with this study's findings. McIntyre reported a C/P ratio of 0.83 and an L/P ratio of 1.9 which are lower than the averages from this study, but still suggests that mitragynine does not undergo PMR. The differing number of data points in each study could contribute to the differences in ratios, as well as the postmortem interval (PMI) which is the time elapsed between death and specimen collection at autopsy. The PMI is not known or well-documented in the case studies presented and the other publications referencing PMR. The PMR can increase as the PMI increases, which could cause the differences between data sets.

Limitations

There are several limitations to the results in this study which should be considered. The scope of this project only included two alkaloids for kratom including mitragynine and 7-OH. 7-OH was positively identified and not quantitated because of the aforementioned issues with stability, its small abundance in the plant material, and apparent small abundance in postmortem

specimens. There are several diastereomers for mitragynine which could also be identified but have not been heavily researched up to this point. A potential limitation of the stability study is that it only stretched over 90 days. The 90-day timeline was prioritized for the OCME because case backlog could delay analysis up to 90 days from specimen collection at autopsy. Other stability studies for mitragynine and 7-hydroxymitragynine were typically the same amount of time or shorter including Papsun et al. at 90 days. There was a one-year study completed in antemortem and postmortem blood along with urine, liver and brain (Kedzierski, 2022). The stability study in this project utilized control blood as a matrix match but did not assess stability in postmortem blood or other matrices. Since postmortem specimens have a complex matrix, using negative postmortem matrix would give more comparable results for the stability study. The degree of postmortem matrix breakdown between decedents is something that cannot be easily simulated.

The case study assessed ten real world cases which spanned over one year. More case studies over a larger time period would give a more accurate depiction of typical postmortem concentrations in heart and femoral blood as well as liver. The case studies did not utilize any matrix other than blood and liver, which limits the information about distribution of the postmortem concentration to other matrices such as vitreous, urine, or other tissues. Blood and liver are the matrices typically used for quantitative analyses in the OCME, which is why they were chosen for the study.

Suggestions for Further Research

The legality of kratom is rapidly changing over time all over the world. The rising popularity and legality of kratom could cause a steady rise in its usage as well as spiked products. More kratom alkaloids could rise in popularity and the studies on them are lacking. Stability studies for

other kratom alkaloids will most likely need to be accomplished as well as longer lasting studies on mitragynine and 7-hydroxymitragynine. Stability studies utilizing postmortem blood should be completed in the future to determine the effect of postmortem artifacts and pH on mitragynine and 7-hydroxymitragynine. Since mitragynine had worse stability at -30°C than at 4°C, the stability at temperatures between the two should be accomplished to determine at which temperature mitragynine is most stable.

The evaluation of more case studies would be suggested. A larger body of data regarding postmortem concentrations of mitragynine will aid toxicologists and pathologists in the interpretation of cases and possible cause of death. Other matrices could also be assessed to determine the postmortem distribution of mitragynine all over the body. Since there is a general lack of literature regarding mitragynine and other alkaloids, especially in forensic science, more experimentation would help expand the body of knowledge.

Conclusion

A method was validated for the quantitation of mitragynine and the qualitative identification of 7-OH in blood and liver samples. The stability of mitragynine and 7-OH was assessed for three days at ambient temperature and up to 90 days at refrigerated and frozen temperatures in blood. Ten different real world case studies were analyzed determining the postmortem mitragynine concentration in heart blood, femoral blood, and liver to evaluate distribution of mitragynine.

The validation of the method was needed to complete the stability project and case studies and also will be utilized by the OCME lab in regular casework. The validation criteria for mitragynine and 7-OH were met according to the ASB standards. There is not much data including long stability studies in several different storage conditions for mitragynine and 7-OH,

so this study will aid in adding to the established literature. Stability data is needed in forensic chemistry and toxicology in order to generate accurate and reliable results. It was found that mitragynine had less degradation in refrigerated conditions than the frozen conditions. The refrigeration of blood samples will help mitragynine be more stable before quantitation takes place. 7-OH was not as stable in refrigerated conditions, but the instability of the drug was already known prior to the project. The drug will only be qualitatively identified in casework because of this instability.

The case studies will help the OCME lab in the future to identify the range of mitragynine concentrations in postmortem cases. Case studies are needed so that a range of lethal concentrations can be established to aid in interpretations of the case. The toxicologists and pathologists use the postmortem concentrations of the drugs present to interpret a case and determine the probable cause and manner of death. The C/P and L/P ratios were also assessed to add to the literature in order to determine if mitragynine undergoes postmortem redistribution. If a drug is known to undergo postmortem redistribution, then femoral blood will most likely be utilized for a quantitation if it is available, so the postmortem concentration is a better representation to the concentration in the blood at death.

The data from the case studies adds to the limited body of knowledge regarding mitragynine's role in medicolegal death investigations and aids the forensic toxicologist's and pathologist's interpretation of mitragynine positive cases. Timely and accurate reporting from the OCME allows closure of a death investigation, confidence in the practice of forensic medicine and provides the decedents' family peace of mind.

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