DEVELOPMENT AND VALIDATION OF TOXICOLOGICAL METHODS FOR COGNITIVE STIUMLANTS IN TRADITIONAL AND ALTERNATIVE MATRICES

_____

A Dissertation

Presented to

The Faculty of the Department of Forensic Science

Sam Houston State University

_____

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

_____

by

Christina Renee Smith

December, 2021

DEVELOPMENT AND VALIDATION OF TOXICOLOGICAL METHODS FOR COGNITIVE STIUMLANTS IN TRADITIONAL AND ALTERNATIVE MATRICES

by

Christina Renee Smith

_____

APPROVED:

Madeleine J. Swortwood, PhD
Committee Director

Sarah Kerrigan, PhD
Committee Member

J. Tyler Davidson, PhD
Committee Member

Erin Karschner, PhD
Committee Member

Phillip Lyons, PhD
Dean, College of Criminal Justice

DEDICATION

"How am I ever gonna get to be old and wise if I ain't ever young and crazy?"

-Frankie Ballard

To my dad.

In the last conversation we ever had, I told you I would get my PhD. Guess what? I did it. Your resiliency and tough love (and shared tolerance for C_2H_5OH) bled throughout my entire journey. Without that, I wouldn't be where I am today. To the cornerstone of our family, thank you.

ABSTRACT

Smith, Christina Renee, *Development and validation of toxicological methods for cognitive stimulants in traditional and alternative matrices*. Doctor of Philosophy (Forensic Science), December, 2021, Sam Houston State University, Huntsville, Texas.

Attention-deficit/hyperactivity disorder (ADHD) is a neurological disorder that arises from a lack of dopamine in the brain. Patients with this disorder have an increased level of dopamine reuptake transporters in the brain which leads to a lack of dopamine in the synapse. The dopamine deficiency leads to inability to pay attention, lack of focus and boredom. Medications work to combat this disorder by blocking dopamine reuptake transporters thus increasing dopamine and speeding up brain activity. One of the main medications prescribed to treat ADHD is methylphenidate. Methylphenidate has two chiral centers which gives rise to four stereoisomers: the *threo*- and *erythro*-configurations of the *dextro*- and *levo*-methylphenidate enantiomers. The *threo*-methylphenidate configuration is known to be responsible for the pharmaceutical effects, specifically the *d* enantiomer as the *l* enantiomer has been proven to be toxic. Methylphenidate is typically sold as a racemic mixture of *threo*-methylphenidate with both the *d* and *l* enantiomer present. Due to the differing effects of the enantiomers, it is important to separate the enantiomers to better understand the pharmacodynamic and pharmacokinetics (PD/PK) of these medications. Methylphenidate is metabolized to ritalinic acid and, in the presence of ethanol, can break down to ethylphenidate. There are minimal comprehensive methods that separate the enantiomers of methylphenidate and its metabolites. To bridge the gap in knowledge, this study aims to analyze these cognitive stimulants in traditional and alternative matrices across multiple analytical

platforms. Additionally, stability of these analytes needs to be assessed to better understand proper handling conditions of forensic toxicology specimens.

To better understand cognitive stimulants, such as methylphenidate, this study sought to develop analytical methods that can be used for the quantification of these analytes. Additionally, proof of applicability was conducted to demonstrate method validity. The main goals of this study were to 1) develop and validate a method for the chiral separation and analysis of *d,l*-methylphenidate, *d,l*-ethylphenidate and ritalinic acid in blood using liquid chromatography-tandem mass spectrometry (LC-MS/MS); 2) develop and validate an achiral method for *d,l*-methylphenidate, *d,l*-ethylphenidate, lisdexamfetamine, and amphetamine in oral fluid using LC-MS/MS with application of the method to authentic oral fluid samples; 3) develop and validate a method for the chiral separation and analysis of *d,l*-methylphenidate, *d,l*-ethylphenidate and *d,l*-ritalinic acid in blood using supercritical fluid chromatography (SFC) coupled to LC-MS/MS and apply the method to authentic postmortem blood samples; and 4) assess long- and short-term stability of *d,l*-methylphenidate, *d,l*-ethylphenidate and ritalinic acid in blood.

A method was developed, optimized and validated for quantification of *d,l*-methylphenidate, *d,l*-ethylphenidate, and ritalinic acid in blood using LC-MS/MS. Chiral separation of the enantiomers was achieved using an Agilent Chiral-V column and this method was considered acceptable per validation guidelines with the exception of ion suppression/enhancement. However, the deuterated internal standards compensated for this as well as reproducibility of the effects. This method proved to be suitable for chiral separation without the need for hazardous and costly derivatizing agents traditionally used for separating enantiomers.

A method was developed, optimized, and validated for quantification of methylphenidate, ethylphenidate, lisdexamfetamine and amphetamine in oral fluid using LC-MS/MS. This method was considered sensitive and acceptable per validation guidelines except for ion suppression/enhancement. Similar to the blood method, the deuterated internal standard compensated for this phenomenon. For proof of applicability, this method was applied to 4 authentic oral fluid samples collected from college students alongside self-reported medication use. Both lisdexamfetamine and amphetamine were detected in the samples, as expected from subject surveys. This method demonstrates that oral fluid can be used as an alternative forensic toxicology matrix for detection of cognitive stimulants.

A method was developed and optimized for quantification of *d,l*-methylphenidate, *d,l*-ethylphenidate and *d,l*-ritalinic acid in blood using SFC-MS/MS. Method validation was conducted and was deemed acceptable. This method was applied to 49 authentic postmortem samples in which the enantiomers of the analytes were quantified and compared to results achieved from an achiral assay. Of the 49 samples, *d,l*-ritalinic acid was detected in all 49 samples, *d*-methylphenidate was detected in 29 samples, *l*-methylphenidate was detected in 15 samples, *d*-ethylphenidate was detected in 5 samples and *l*-ethylphenidate was detected in 1 sample. This technique offers an alternative way to achieve chiral separation of analytes.

Lastly, the stability of *d,l*-methylphenidate, *d,l*-ethylphenidate, and ritalinic acid were assessed over a 9 month period. Storage under frozen temperatures (-20ºC) was the only condition in which all analytes remained stable. A follow up study was conducted to assess methylphenidate degradation and determined that methylphenidate degrades to

ritalinic acid under non-frozen conditions. This study demonstrates the importance of understanding proper sample handling and storage conditions as well as time of analysis for unstable drugs where quantification may be of important toxicological value.

The developed analytical methods herein offer chiral separation and quantification of methylphenidate and other cognitive stimulants in blood and oral fluid through various analytical techniques. As the potential for cognitive stimulant abuse and misuse is rising, it is important to analyze these analytes in forensic toxicology samples. Data from these studies can be useful for laboratories to better understand chiral analysis, alternative matrices and stability of these analytes for proper detection, quantification, and interpretation.

KEY WORDS: Chiral separation, Methylphenidate, Cognitive stimulants, LC-MS/MS

ACKNOWLEDGEMENTS

To my mom: My rock. My bestfriend. Thank you for always being there when I needed it: to laugh, to cry, to dog sit, to help me find things in the grocery store. Thank you for the never-ending support through this all. Your love is what got me through, even the hardest days. I hope I can be half the mom and woman you are one day.

To Brandi: Thank you for always being completely insane with me – all the time. I have always felt your support over the years. Thankful I got you as a sister and not anyone else.

To Riley: the best dog there ever was.

To my advisor and mentor, Dr. Swortwood: I am forever indebted to you for your compassion towards me during graduate school, both in and out of the walls of CFS. Your patience, support, and dedication to me is something I am forever thankful for. Not only have you become a life-long mentor but also a friend.

To my lab partner and bestfriend, Dr. Kaitlyn Palmquist: Words cannot express what these years have meant to me. Thankful that MD brought you to TX. Thankful that we have so many laughs (a lot of laughs) and memories to share. Thankful that we have the same love for happy hour and football. I would have simply not graduated if I did not have you. We did it.

To my Wolfgang (Morgan, Norris, Clarra).

To Dr. Michael Truver: “thank you for being my lab buddy and the late night Facetimes.”

To Allyson Valerius: I could not have finished undergrad without you, and I would not have finished graduate school without you. Thankful we are cut from the same cloth.

To Emily Deyoung, J.D.: Remember when all people thought all we would ever be was just mean girls? They should see us now.

To the families who have loved me like their own: The Fisher's (6.22, for life). The Davis's. The Jacob's. The Arrington's.

To the places that felt like home: Loebau, TX. Hampton Inn and Suites. White Rhino Coffee. 612 Hickory St.

To the friends who never wavered: Tori. Hailey. Overbay. Taylor. Brenda. Jan. Brandon. Callie. Kelsey. Haylie. Kasey.

To Sam Houston State University – Forensic Science and Dr. Kerrigan.

To the agave plant.

To my Lord and Savior, Jesus Christ.

Thanks and Gig 'Em, forever.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER I

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is the most common neurobehavioral diagnosis for children under 18 year of age (1). Globally, ADHD affects 1.5-5% of the population with approximately 3.5% of children and 1.4-3.6% of adults, respectively, being diagnosed (2-4). ADHD is characterized by three core symptoms: hyperactivity, impulsivity and inattention. These symptoms can be exhibited as boredom, lack of focus, or difficulty hearing or focusing. Patients can be classified as predominantly hyperactive/impulsive or predominantly inattentive. There is occasionally a third subclass which combines the first two (1, 5-7). The symptoms by subclass are summarized in Table 1.1.

Table 1.1. Subclasses of attention-deficit/hyperactivity disorder and the symptoms commonly associated*

Subclasses of ADHD	*Symptoms associated*
Inattention	• Lack of attention to detail – commonly makes mistakes • Lack of sustained attention • 'Not listening' when being spoken to • Inability to finish chores and tasks – not organized • Losing things that are necessary for tasks • Easily distracted • Forgetful in daily tasks
Hyperactivity/impulsivity	• Constant fidgeting with something, tapping hands or feet, squirming, pacing, inability to sit still • Feeling restless or agitated • Lack of engagement in leisure activities quietly • Always 'on the go' • Excessive talking • Difficulty waiting one's turn • Interrupting or intruding others
Areas of impairment	*Examples associated*
Health problems, psychiatric comorbidities	• Learning or coordination disorders • Speech or language disorders • Mood, anxiety, tic disorders • OCD or autism scale • Overweight, obesity, metabolic disorders • Accidents (including driving) and safety issues • Suicidal ideations
Functional impairment	• Low self-esteem (undetermined sense of well-being) • Unable to regulate emotions
Academic or occupational challenges	• Underperformance • Being held back in school or repeating classes – related to inattention • Special education needs • School expulsion or drop out
Social/interpersonal problems	• Inadequate social skills • Long term issues with family/intimate relationships

*Table 1.1 adapted from Jaeschke et al. (8)

Studies demonstrate that ADHD is expressed in genders differently. Males are three times more likely to be diagnosed with ADHD. Males are more likely to display symptoms that align with the hyperactive/impulsive subclass with aggressive behaviors and a higher probability of having problems with the law (9, 10). Females are more likely to fall into the inattentive subclass and potentially struggle with mental health and eating disorders (10). Though typically diagnosed in children, ADHD has a neurodevelopmental origin that can manifest into adulthood (11-14) with 60-80% of ADHD symptoms persisting in adults (9). Though some studies show that only 1.4-3.6% of adults are affected by ADHD, other studies have stated that this number may be closer to 4-4.5% (15, 16). Starting at a young age, children with ADHD display difficulty in forming relationships due to social anxiety, aggressive attitude, and impulsive behavior. School-age children display slower rates of processing information which leads to poor academic performance, including low standardized test scores and grades. Studies show these students also have a higher chance of dropping out of school (9, 17). As children enter adolescence, patients with ADHD express low self-esteem that can strain social relationships or increase chance for substance abuse or problems with the authority, such as law enforcement (9). Moving into adulthood, ADHD symptoms can manifest into poor job performance, lower socioeconomic status, and prolonged social issues, especially in marriages or relationships (18). To avoid issues in adolescence and adulthood, treatment and diagnosis during childhood are important (19). However, diagnosis of this condition is difficult due to varying symptomology of each subclass. For diagnosis within a subclass, patients must display a minimum of six symptoms (5 if over the age of 17) for longer than 6 months in addition to functional impairment in certain settings (1, 7, 20). In

addition to the criteria, ADHD can be underdiagnosed due to overlap of symptoms with other disorders such as anxiety, borderline personality disorder, oppositional defiant disorder, or multiple personality disorder (21, 22). For proper diagnosis, clinicians need to understand the specific area of impairment for their patient to determine the best treatment (8).

To understand treatment, the neurology behind ADHD must first be understood. Parts of the brain responsible for ADHD are the prefrontal cortex, caudate and cerebellum. These parts of the brain are responsible for the regulation of emotion, behavior, thoughts, and attention. With ADHD, these parts of the brain display reduced activity or immature formation (23, 24). These areas of the brain are maintained by neurotransmitters (NT), such as dopamine (DA) and norepinephrine (NE), that interact with each other utilizing the receptors or transports on the ends of the neurons (25-28). ADHD comes from a reduced amount of DA receptors in various brain regions (17). Research indicates that MPH binds to the dopamine transporter in the pre-synaptic cell which inhibits DA from being taken back into the cell, thus creating an increase in DA and leading to quicker brain activity (29-33). Studies show that ADHD may arise from a polymorphism in the genes that code some DA receptors and transmitters which reduce functionality (34). Ultimately, for optimal brain performance, there needs to be a balance of DA and NE. With imbalance, ADHD arises. Medications are prescribed to balance these NTs in the brain.

ADHD Treatment

There are two common ways that ADHD is treated: drug therapy or behavioral therapy (or a combination of the two). Treatment early in childhood reduces issues and

manifestations that may persist into adulthood (9). In many cases, drug therapy is more effective than behavioral therapy. In 1999, there was a study conducted called the Multimodal Treatment Study of Children with ADHD that evaluated the two treatments for ADHD (35). This study confirmed that drug therapy is more effective than behavioral therapy in controlling ADHD symptoms and that there is no additive effect when both therapies are being practiced. In other studies, it has been observed that behavioral therapy is just as effective as low dosages of stimulants, making it preferable (7). The type of therapy conducted is selected based on each patient and the severity of their symptoms and needs.

The Food and Drug Administration (FDA) has approved two types of medications to treat ADHD via drug therapy: stimulants (methylphenidate and amphetamine) or non-stimulants (atomoxetine and extended-release α_2 agonists (guanfacine and clonidine)) (19). Table 1.2 summarizes stimulant and non-stimulant medications and adverse side effects associated with them. Both stimulant medications come in immediate- and extended-release forms with equal efficacy (36, 37). Though extended-release tablets are more expensive, they prove to be more beneficial regarding convenience, confidentiality at work or school, compliance, driving performance, and reduced potential for abuse (38-41). In the case that neither stimulants or non-stimulants can be used, tricyclic anti-depressants, immediate-release α_2 agonist, and bupropion have all been used as off-label ways to treat ADHD (19). As the stimulants are the main interest of this study, those are the only class of treatments that will be discussed in detail.

Table 1.2. A summary of formulations, strengths and adverse effects of stimulant and non-stimulant medications used to treat ADHD

Brand Name (Generic) *Duration of Action*	*Dosages available*	*Adverse effects*
	Stimulant	
Adderall® (amphetamine and dextroamphetamine salts) 4-6 hours	5, 7.5, 10, 12.5, 15, 20 30 mg IR tablet	Insomnia, anorexia, abdominal pain, weight loss, headache, irritability, emotional liability, anxiety, increased blood pressure, dry mouth, nausea, vomiting, diarrhea
Adderall XR® (amphetamine, dextroamphetmine salts) 10-12 hours	5, 10, 15, 20, 30 mg ER capsule	
Vyvanse® (lisdexamfetamine) 12 hours	20, 30, 40, 50, 70 mg Capsule	
Dexedrine® (dextroamphetamine) 4-5 hours	5, 10 mg IR capsule 5, 10, 15 mg ER capsule	
ProCentra® (dextroamphetamine) 4-5 hours	5mg/5mL Oral solution	
Ritalin® (methylphenidate) 3-4 hours	5, 10, 20 mg IR tablet	
Methylin™ (methylphenidate) 3-4 hours	5, 10, 20 mg IR tablet 2.5, 5, 10 mg Chewable tablet 5mg/5mL, 10mg/5mL Oral solution	
Ritalin LA® (methylphenidate) 8-10 hours	10, 20, 30, 40 mg ER capsule	
Ritalin SR® (methylphenidate) 8 hours	20 mg ER tablet	
Concerta® (methylphenidate) 12 hours	18, 27, 36, 54 mg ER tablet	
Metadate CD® (methylphenidate) 8 hours	10, 20, 30, 40, 50, 60 mg ER capsule	
Metadate ER® (methylphenidate) 8 hours	20 mg ER tablet	
Methylin ER™ (methylphenidate) 8 hours	10, 20 mg ER tablet	
Daytrana® (methylphenidate) Dependent	10mg/9 hr, 15mg/ 9hr, 20mg/9 hr, 30mg/9 hr Transdermal patch	
Quillivant XR® (methylphenidate) 12 hours	25mg/5mL ER powder for suspension	
Focalin® (dexmethylphenidate) 6 hours	2.5, 5, 10 mg IR tablet	
Focalin XR® (dexmethylphenidate) 12 hours	5, 10, 15, 20, 25, 30, 35, 45 mg ER capsule	

(continued)

Brand Name (Generic) *Duration of Action*	*Dosages available*	*Adverse effects*
	Non-stimulant	
Strattera (atomoxetine) 10-12 hours	10, 18, 25, 40, 60, 80, 100 mg IR capsule	Decreased appetite, nausea, vomiting, fatigue, insomnia, abdominal pain, dry mouth, constipation, somnolence, urinary retention, dysuria, erectile dysfunction, dysmenorrhea
	α_2 agonists	
Kapvay® (clonidine) 4-6 hours	0.1 mg ER tablet	Somnolence, fatigue, upper-respiratory tract infection, dry mouth, bradycardia, irritability, midsleep awakenings, sore throat, insomnia, nightmares, constipation, increased body temperature, ear pain, nausea, lethargy, dizziness, hypotension, headache
Intuniv® (guanfacine) 24 hours	1, 2, 3, 4 mg ER tablet	
	Bupropion	
Wellbutrin (bupropion) 6-8 hours	75, 100 mg IR tablet	Dizziness, tachycardia, anorexia, constipation, nausea, vomiting, irritability, sedation, rash, weight gain/loss, impotence, menstrual complaints, dry mouth, akinesia, bradykinesia, abnormal dreams, hyperhidrosis, headache, migraine, insomnia, tremor, agitation, confusion, hostility, fatigue, upper-respiratory complaints, blurry vision, auditory disturbance, anxiety, impaired concentration
Wellbutrin SR (bupropion) 8-12 hours	100, 150, 200 mg SR tablet	
Wellbutrin XL (bupropion) 24 hours	150, 300 mg ER tablet	
	Tricyclic antidepressants	
Norpramin® (desipramine) 8-12 hours	10, 25, 50, 75, 100, 150 mg IR tablet	Hypotension, hypertension, palpitations, heart block, myocardial infarction, stroke, arrhythmias, tachycardia, ventricular fibrillation, sudden death, hallucinations, disorientation, delusions, anxiety, restlessness, agitation, nightmares, numbness, paresthesia of extremities, ataxia, tremors, extrapyramidal symptoms, peripheral neuropathy, dry mouth, blurred vision, urinary retention, suicidal ideation, manic episode, insomnia, panic attacks, constipation, nausea, vomiting, anorexia, diarrhea, dysgeusia, heartburn, weight gain, hyperhidrosis, erectile dysfunction, ejaculation dysfunction
Tofranil(TM) (imipramine) 8-12 hours	10, 25, 50 mg IR tablet	

Abbreviations: extended release (ER), immediate release (IR); table adapted from Sharma et al. (19)

Stimulants

Methylphenidate (MPH) and amphetamine (AMP) are both Schedule II drugs under the Controlled Substances Act in the United States due to their use in medical settings but potential for abuse. Both of these types of stimulants are approved by the FDA for treatment of ADHD at all age groups (20). Effective doses, per FDA guidelines, are typically exceeded in adults to achieve appropriate response. However, there is little data to understand the safety and efficacy of these higher doses (43). Stimulants work to inhibit DA and NE reuptake in the brain via inhibition of the dopamine transporter (DAT) and norepinephrine transporter (NET). Amphetamine has the capability to enter the presynaptic terminal via DAT and NET and release DA and NE into the synapse (44-46). In addition, these medications inhibit monoamine oxidase, the enzyme that metabolizes these NTs (28). Overall, stimulants work to balance DA and NE in the brain.

Issues arise with the safety of long-term AMP or MPH use as they are ranked 8th and 13th for substances known to cause dependence and 6th and 12th for substances known for causing physical harm, respectively (47). There are also concerns with the fairness of stimulant use in school performance due to an increase in memory function which, in turn, would increase academic performance (48). On the other hand, there are reports that long-term use of these medications lead to memory impairment (24). Lastly, research indicates that these stimulants affect physical growth (36, 49-51). Despite these concerns, both MPH and AMP have proven to be effective for long-term ADHD treatment (39). When taken as prescribed, there have been no data to suggest inhibition of DAT and NET in the area of the brain that is responsible for reward (52), reducing the risk of dependence. In fact, ADHD treatment with these medications, when initiated at a young

age, reduces risk of substance abuse later in life (53-55). Other long-term effects of these medications are decrease in emergence of other disorders (such as anxiety, oppositional defiant disorder (ODD), etc.), reduction of aggressive behaviors, and decreased social anxiety (56, 57). Though these stimulants work by increasing the ability to concentrate for academic performance, these medications do not enhance learning or improve academic and occupational performance in children and adults, respectively, in an unfair way (58, 59). Cognition impairment has only been reported in animals (60) and is not problematic if taken as prescribed in ADHD patients (19). Overall, these medications work to limit the effects and symptoms of ADHD without enhancing other effects in the body.

Methylphenidate

As this stimulant is the focus of this study, this medication will be discussed in detail in the following sections. The most common prescriptions that contain both enantiomers of MPH (racemic) are Ritalin®, Concerta®, Metadate® and Daytrana®. The main prescription that contains only *d-threo*-MPH (the most active enantiomer) is Focalin® (20). Formulations include transdermal patches (provides greater bioavailability) (7) and immediate- and extended-release oral dosages at various strengths. MPH works to increase DA concentrations while DA is being actively released in the brain, such as during complex cognitive actions (61). When comparing MPH to AMP, MPH has a reduced risk of dependency and potential to cause physical harm (47) due to slower uptake, slower clearance and reduced potential for drug-drug interactions (62, 63).

Amphetamine

The most commonly prescribed amphetamine-based medications are Adderall XR® (amphetamine) and Vyvanse® (lisdexamfetamine) (19). Both isomers of amphetamine are active. Amphetamines are metabolized by CYP1A2, CYP2C9, CYP2D6, and CYP3A4 (63). Due to CYP2D6 genetic polymorphisms, metabolic rates vary among individuals (slow vs. fast metabolizers) and amphetamine concentrations may be increased in certain ethnicities (39). Both prescriptions have risk for abuse, dependency and toxicity if not used as prescribed (64). Adderall XR® is more likely to be abused than Vyvanse® due to quicker release and absorption of amphetamine which increases euphoria (40, 41).

Non-stimulants

Stimulants are not suitable for 30% of patients being treated with ADHD (65, 66). Lack or minimal response to medications, intolerable side effects, medical disorders and family medical history, or familial avoidance of stimulants are all reasons stimulants are not effective or desired for treatment (19). Non-stimulants are prescribed mainly as Atomoxetine (Strattera), extended-release α-2 agonist clonidine (Kapvay®) and guanfacine (Intuiniv®). These medications are not discussed in detail herein as they are not the focus of this study, but their formulations and adverse effects are summarized in Table 1.2. When comparing stimulants (MPH) to non-stimulants (atomoxetine), there are no direct comparison studies and all data are based on meta-analyses (7, 39, 40, 67, 68). Overall, stimulants are typically favored for treatment due to efficacy in managing symptoms. However, some studies have shown that stimulants and non-stimulants are equally effective. Discrepancies may be due to differences in study methodology or

outcome measurements. Additionally, many of these studies or analyses were conducted over a short period of time, but non-stimulants take weeks to reach their full effects (69-71). Further studies need to be conducted to address efficacy and effectiveness when comparing stimulant and non-stimulant medication treatment therapies. Ultimately, each patient requires individual assessment as responses to medications or treatments vary among the individual. Clinicians are responsible for understanding each patient suffering with ADHD and choosing the best plan of treatment based on their symptoms, severity, and physical or mental needs.

Abuse of Cognitive Stimulants – College Students

As already mentioned, ADHD is not a disorder only observed in children. Symptoms of ADHD can persist and manifest into adulthood adding strain on relationships, academic or job performance, and behavioral control. ADHD is the fastest growing disability on the college campus (72). At the collegiate level, this disorder typically manifests as increased anxiety, mood disorders and poor performance in school. To combat this, stimulants are usually prescribed to this population to help increase alertness, awareness, and the ability to pay attention. However, stimulants do not increase ability to learn and apply knowledge (57, 58, 73-79), they only work to help individuals focus on tasks at hand and improve their listening skills.

The mainstay medications prescribed to treat ADHD in adults are MPH, AMP, and lisdexamfetamine with MPH being the most prescribed (19, 80-84). Studies indicate that 3-5% of college age students are prescribed stimulants and approximately one-third of those students admit to giving or selling their prescribed medication to someone else for non-medical use (85). This displays the potential for misuse and abuse of these

stimulant medications at the college level. From 2003-2017, the National Survey on Drug Use from Monitoring the Future reported that misuse of methylphenidate among college students increased from 1.4 to 5.7% over this time frame (86). Wilens et al. conducted a review of the literature and concluded that stimulant misuse among college students ranged anywhere from 5-35% (87). Numerous other studies also report misuse and abuse of cognitive stimulants across college campuses. McCabe et al. conducted a survey among 9,000 undergraduates and found that 5% of students misuse stimulants and often co-abuse other substances (88). McCabe et al. published an additional study where 10,000 students across 119 colleges were asked to self-report medication use. Lifetime non-prescribed stimulant use among this population was 6.9%. Among this population, past-month stimulant use was 2.1% (89). Their findings indicate that white male students who participate in a panhellenic organization (fraternity/sorority) or students with low grade point averages were among those with the highest use/misuse (89). Babcock et al. analyzed a group of 283 students and 17% reported MPH misuse (90). Low et al. surveyed a group of 150 undergraduates and found that 35% misused MPH or AMP without a prescription. Among those, 80% claimed the misuse was for performance enhancement. When compared to students who did not report misuse, those who misused stimulants also reported illicit stimulant use such as cocaine or ecstasy (91). White et al. used a web-based survey to determine that 16% of undergraduate students (out of 1,025) misused stimulants (92). Lastly, 1.3% of students ranging from 18-25 years of age had misused ADHD stimulants in a study conducted by Kroutil et al. (93). Other studies conducted analyze the illicit distribution of stimulant medications at the college level. Wilens et al. analyzed a group of 98 adults with ADHD (mean age – 21) and found that,

within the last 4 years, 11% of individuals had sold their prescribed medications. Among this group, people who sold their medications were also more likely to misuse their prescription (94). In another study, McCabe et al. found that 23% of the college age respondents were asked by someone else to distribute their medications for non-prescribed use (95). Lastly, Upadhyaya et al. surveyed 334 college students and found that 29% have sold their prescribed ADHD medication (96). These data indicate that stimulant use is a concern among college age students. With accessibility increasing in this population, there is a need for better monitoring of these medications. Misuse and abuse of these medications can lead to unwanted and adverse effects, especially when taken for non-medical purposes.

Methylphenidate

Prevalence

Methylphenidate is the most commonly prescribed psychostimulant to treat ADHD (97). Due to its high potential for abuse, questions arise around the concern of its illicit use (98). MPH misuse has increased among adolescents in the past decade. As stated previously, there was also an increase in methylphenidate use among college campuses (86) as indicated by several surveys into MPH misuse and distribution on college campuses. As MPH has approved medical use and is a risk of abuse, it is imperative for forensic toxicologists to detect these analytes in various matrices. MPH should be routinely screened for and methods are needed for drug detection and quantification in traditional and alternative forensic specimens.

Pharmacology

MPH, belonging to the class of phenylethylamines, was first synthesized in 1944 and has the chemical name methyl-2-phenyl-2-(piperidin-2-yl)-acetate (Figure 1.1) (99, 100). MPH has two chiral centers which produces four stereoisomers with *d-threo*-MPH being the most active (see chirality section). MPH acts as an agonist against the DAT and NAT (99, 101) and its pharmacological activity comes from the direct inhibition of these transporters. The main mechanism of MPH is to boost the dopaminergic transmission in the brain by extending the activity of DA in the synapse. MPH has been shown to block >50% of DAT in the brain which increases DA in the space. When ADHD is being treated with MPH, the number of available binding sites for DA is reduced which alleviates the symptoms of ADHD (8, 102, 103). Studies show that MPH can produce a 3-4 fold increase of DA and NE in the brain (104). MPH has the highest affinity for DAT as compared to NAT. MPH does not have any effect on the serotonin transporter (SERT) (105-107). When comparing to SERT, MPH has a 2200 times higher affinity for DAT and a 1300 times higher affinity for NAT (108). Additionally, MPH works to increase the dopaminergic activity in the brain which increases central nervous system function which leads to for both behavioral and cognitive changes (109).

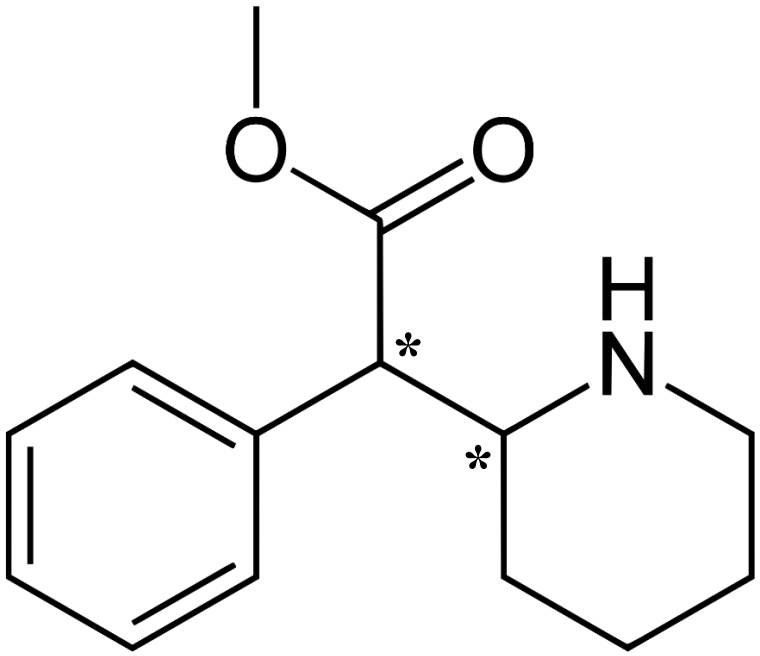

*Figure 1.1. Structure of methylphenidate (chiral centers indicated by *)*

Chirality

As stated, MPH has four configurational isomers. Due to two chiral centers, there are four stereoisomers of MPH: *erythro* [(*d*-(2R:2'R) and *l*-(2S:2'S)] and *threo* [[(*d*-(2R:2'R) and *l*-(2S:2'S)] (Figure 1.2) (8). When MPH was first synthesized, the original formulations were racemic mixtures of 80% *d,l-erythro*-MPH and 20% *d,l-threo*-MPH (110-112). It was later discovered that the stimulant effect comes from the *threo*-MPH isomer. Since then, medications were formulated as a racemic mixture of *d,l-threo* MPH (112, 113). Additionally, it has been shown that the *d* enantiomer, specifically, is responsible for pharmaceutical effects of this medication (112, 114, 115). As such, more recent prescription drug formulations only contain *d-threo*-MPH which is the most potent form (112, 114). In the brain, isomers distribute differently (116). The *d* enantiomer has

the highest affinity for DAT and NAT (giving it the pharmaceutical effect) while the *l* enantiomer has the lowest binding affinity on DAT and NAT (106, 117, 118).

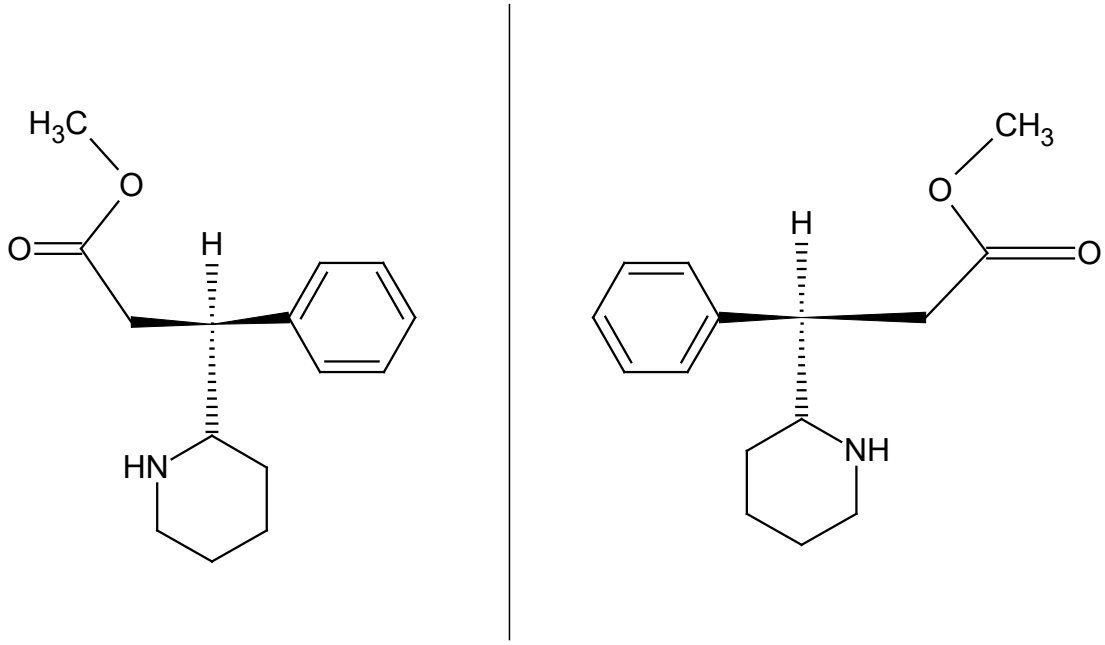

Figure 1.2. The enantiomers of MPH (left: l-threo-methylphenidate; right: d-threo-methylphenidate)

Pharmacokinetics

After oral ingestion of MPH, it is quickly absorbed from the gastrointestinal tract and buccal mucosa (82) and undergoes large first pass metabolism effects. Bioavailability in humans is low ranging from 11-53% (82, 100, 112). MPH is highly lipophilic and exhibits low protein binding (15%), so it is rapidly distributed into the tissues (Volume of distribution: 5-6 L/kg) (45, 82, 111, 114, 119). Studies indicate that MPH concentrations in the brain are 8x higher than blood (82). MPH has a short half-life (2-3 hours) with 50% of MPH is excreted in urine within 8 hours of administration with 90% clearance within 48 hours post-administration. (120). Only <1-2% of MPH is unchanged in urine (108, 113). MPH is metabolized in the liver by endoplasmic reticulum human carboxylesterase 1A1 (CES1A1) to its inactive metabolite, ritalinic acid (RA) (Figure 1.3) through de-

esterification (113). This is an enantioselective process as CES1A1 has a higher affinity for *l*-MPH than *d*-MPH which leads to an increase of *d*-MPH plasma concentrations and longer half-life (114). In mice, hydroxylated metabolites produce pharmacological effects but not in humans (100). Another animal study showed that para-hydroxy-MPH undergoes de-esterification and glucuronidation to produce para-hydroxy-ritalinic acid glucuronide (8). If MPH undergoes microsomal oxidation, 6-oxo-MPH (an inactive metabolite) may be formed and further metabolized to 6-oxo-ritalinc acid through de-esterification (108). In the presence of ethanol, ethylphenidate (EPH) is produced (see ethylphenidate section). For the purpose of this study, only RA and EPH metabolites will be analyzed.

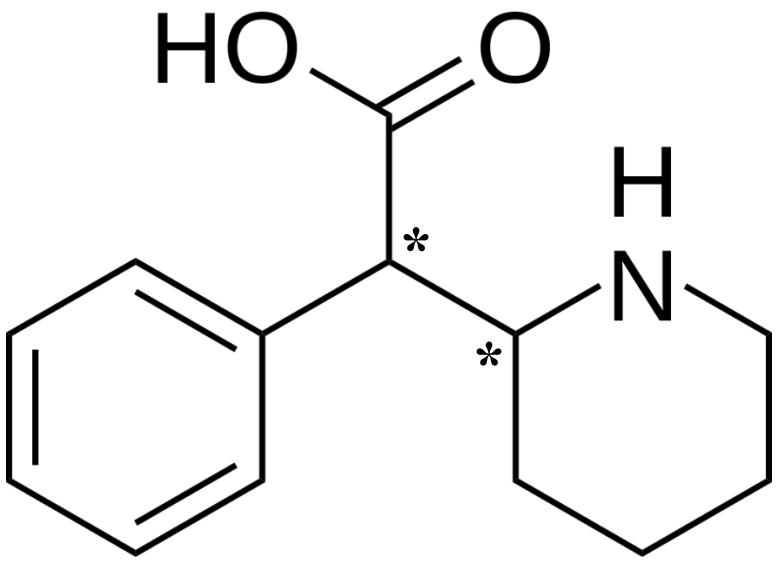

*Figure 1.3. Structure of ritalinic acid (chiral centers indicated by *)*

There are no major differences in the PK patterns of MPH between children and adults (100, 112, 121). However, there are some reports of PK differences between genders that indicate women exhibit lower MPH bioavailability and decreased clearance of MPH in the brain (122, 123). MPH has the potential to interact with other drugs when co-administered with gastric modulators, proton pump inhibitors or H_2 blockers (124). In

addition, when beverages are >40% alcohol, ethanol has contributed to a quicker release of MPH in the extended-release formulations. (113). In a study done by Zhu et al., 14 healthy volunteers (age 22-42) were given *d,l*-MPH (40 mg) or *d*-MPH (20 mg) with or without ethanol (0.6 g/kg) (125). Interactions with ethanol increased the maximum concentration (C_{max}) in plasma of *d*-MPH from *d,l*-MPH by 35%. For the enantiopure *d*-MPH, ethanol increased the C_{max} by 27%. Additionally, MPH also displayed stronger stimulant effects when taken with ethanol (125). MPH can have adverse effects if taken with other drugs or substances. It is important to make sure these medications are taken as prescribed to ensure proper pharmacological activity.

Methods

There are a wide range of published analytical methods that were developed in multiple matrices using various instrumentation. Table 1.3 summarizes methods reported in literature. From an analytical standpoint, MPH has been analyzed most traditionally using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS). MPH has been isolated from biological matrices by protein precipitation, liquid-liquid extraction, dilution, solid-phase extraction, and solid-phase dispersive extraction. The human biological matrices that have been analyzed include hair, urine, blood, plasma, oral fluid, and exhaled breath. Of the methods that have been developed, very few separate the enantiomers of MPH. Enantiomeric separation can occur by using a chiral derivatizing agent, as typically seen in GC analysis. For LC, a chiral column can be used to separate the enantiomers of chiral compounds. These LC columns are packed with a special protein that selectively binds to one enantiomer and allows for separation. Ramos et al. isolated *d,l*-MPH in human

plasma with LLE. The enantiomers were separated using LC-MS/MS with an Astec Chiorbiotic chiral column and a LOQ of 87 pg/mL (129). In another study with plasma, *d,l*-MPH and *d,l*-EPH were isolated by LLE and the enantiomers were separated with LC-MS/MS utilizing an Astec Chirobiotic V2 chiral column (126). The LOQ for this method was 0.025 ng/mL. Chiral separation of *d,l*-MPH and *d,l*-RA in blood was also achieved following SPE and separation with a Chiral-AGP column on LC-MS/MS with a 0.5 ng/g LOQ (127). The enantiomers of *d,l*-MPH have also been studied in non-human models such as mouse brain (128), and rat plasma (62) (with *d,l*-EPH) using LC-MS/MS and ultra-fast liquid chromatography-mass spectrometry (UFLC-MS/MS), respectively. The previously mentioned methods on LC-MS/MS all utilized a chiral column to separate the enantiomers of *d,l*-MPH. For some of the methods that analyzed MPH on GC-MS, derivatizing agents were required to detect separate, detect and quantify *d,*l-MPH (130, 132). LLE and protein precipitation (PP) are commonly used to isolate MPH and its metabolites from biological matrices. However, SPE proves to be a better alternative for extraction as it uses less solvent and reduces matrix interferences, making it suitable for LC-MS/MS analysis. There are few methods that separate the enantiomers of MPH, EPH, and RA using SPE and LC-MS/MS. Due to the pharmacological differences of the enantiomers, it is critical to separate these enantiomers in various biological matrices to better understand the activity of these analytes. Table 1.3 summarizes analytical methods in literature that quantify MPH and RA in various human and animal matrices.

Table 1.3. Analytical methods for quantification of MPH and RA in human and animal biological matrices reported in literature

Matrix	Analyte	Chiral Separation Mechanism	ISTD	Extraction	Instrument	Calibration Range (ng/mL)[1]	LOQ (ng/mL)[1]	Other Analytes	Reference
BL	MPH RA		MPH-d9 RA-d10	PP	LC-MS/MS	0.2-30 10-1500	0.2 5		(134)
BL	*d,l*-MPH * *d,l* -RA	Chiral column	*d,l* -MPH-d10 *d,l* -RA-d10	SPE	LC-MS/MS	0.2-500 ng/g	0.5 ng/g		(127)
BL	MPH		Cyclizine	SPE	GC-NPD	100-2000			(135)
PL	**d,l* -MPH	Chiral column	*d,l* -MPH-d9	LLE	LC-APCI-MS/MS	0.087-26.1	0.087		(136)
PL	MPH RA		MPH-d9 RA-d10	PP	LC-MS/MS	0.2-30 10-1500	0.1 2.5		(134)
PL	* *d,l* -MPH	Chiral column	*d,l* -MPH-d3	LLE	LC-MS/MS	0.025-25	0.025	*d,l* -EPH	(126)
PL	MPH			LLE	GC-MS	0.072-18.25	0.072		(137)
PL	MPH		MPH-d9	PP	LC-MS/MS	0.5-100	0.5		(138)
PL	MPH RA		*d,l* -MPH-d4	PP	LC-MS/MS	0.05-20 0.15-60			(139)
PL	MPH		EPH	PP	GC-MS		2		(129)
PL	MPH			LLE	HPLC-fluorescence	1-80	1		(140)
PL	* *d,l* -MPH	Derivatization	*d,l* -MPH-d3	LLE	GC-MS	0.75-100	0.75		(132)
PL	MPH		EPH	SPE	GC-NPD	5-100	2		(131)
PL	MPH			PP	LC-MS/MS	0.035-40			(141)
PL	* *d,l* -MPH * *d,l* -RA	Chiral column	5-hydroxyindole acetic acid	PP	HPLC-UV	25,000-250,000	25,000		(62)
PL (rat)	* *d,l* -MPH *d,l* l-RA	Chiral column	*d,l* -MPH-d9 *d,l* -RA-d10	LLE	LC-MS/MS	1-500	1	*d,l* -EPH	(142)
PL (rat, rabbit, dog)	* *d,l* -MPH	Chiral column	$(C_6H_5)(C_5H_{10}N)CH(COOCD_3)$	LLE	LC-MS/MS	1.1-1087.5	1.1		(143)

(continued)

Matrix	Analyte	Chiral Separation Mechanism	ISTD	Extraction	Instrument	Calibration Range (ng/mL)[1]	LOQ (ng/mL)[1]	Other Analytes	Reference
UR	MPH RA			SPE	LC-MS/MS		0.5		(144)
UR	* *d,l* -MPH	Derivatization	*d,l* -MPH-d5		GC-MS	0-1000	10		(130)
UR	MPH		*d,l* -AMP-d5 and *d,l* -methamphetmine-d5	LLE	GC-MS			22 sympathomimetic amines	(133)
UR	MPH			SPDE	LC-MS/MS	0.5-200	0.5	Methamphetamine	(145)
UR	MPH RA		Levallorphan	SPE	GC-MS				(146)
UR	MPH RA		RA-d5	Dilution	LC-MS/MS	2-150 40-3000	2 40	Amphetamine and 4-hydroxyamphetamine	(147)
UR	MPH RA		Mepivacaine	Dilution	LC-MS/MS	100-1000 nM 500-5000 nM	100 nM 500 nM		(146)
UR	MPH RA		AMP-d6	Dilution	LC-MS/MS	5-5000	100		(148)
OF	MPH		Propranolol	LLE	LC-MS/MS	2.5-90	2.5	Fenproporex, Diethylpropione	(149)
OF	MPH RA		MPH-d9 RA-d10	PP	LC-MS/MS	1-500 0.25-125	0.1 1		(134)
OF	MPH RA		*d,l* -MPH-d9 *d,l* -RA-d10	Dilution	LC-MS/MS	0.5-75	0.5		(150)
OF	MPH RA		*d,l* -MPH-d4	PP	LC-MS/MS		0.7 0.2		(139)
OF	MPH		MPH-d9	PP	LC-MS/MS	0.5-100	0.5		(138)
Exhaled breath	MPH RA				LC-MS/MS		7 pg/filter 4 pg/filter		(139)
Hair	MPH RA		MPH-d9	Stirring	LC-MS/MS	1-100 pg/mg	1pg/mg		(151)
Hair	MPH		MDMA-d5	LLE	LC-MS/MS	0.5-500 pg/mg	0.5 pg/mg		(152)

(continued)

Matrix	Analyte	Chiral Separation Mechanism	ISTD	Extraction	Instrument	Calibration Range (ng/mL)[1]	LOQ (ng/mL)[1]	Other Analytes	Reference
DBS	MPH		MPH-d10	PP	LC-MS/MS	0.2-25	0.2		(153)
Mouse brain	* *d,l* -MPH	Chiral column		PP to SPE	LC-MS/MS	0.5-100	7.5		(128)

Abbreviations: methylphenidate (MPH), ritalinic acid (RA), blood (BL), plasma (PL), urine (UR), oral fluid (OF), protein precipitation (PP), solid-phase extraction (SPE), liquid-liquid extraction (LLE), solid-phase dispersive extraction (SPDE), liquid chromatography-tandem mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC-MS), atmospheric pressure chemical ionization (APCI), nitrogen phosphorus detector (NPD), high performance liquid chromatography-ultraviolent (HPLC-UV)
*chiral separation
[1]units are in ng/mL unless otherwise specified

Cases

MPH and RA were detected and quantified in antemortem and postmortem case samples in literature and summarized in Table 1.4. Though typically analyzed in clinical studies, MPH was also analyzed in forensic settings such as driving under the influence of drugs (DUID) and postmortem autopsy cases. Joseffson et al. conducted a study that analyzed MPH in various matrices in both clinical and forensic settings (134). In the DUID cases, MPH and RA were trace-22 ng/mL and 55-2080 ng/mL in blood, respectively. In the driving cases, additional drugs of abuse were also detected including amphetamine, THC and diazepam (134). In the ten postmortem cases, MPH and RA were trace-95 ng/mL and 15-974 ng/mL in blood, respectively. Similar to DUID, there were additional drugs of abuse detected in these samples (see Table 1.4) (134). In another DUID study, urine samples were tested from drivers who expressed drowsiness and hyperactivity (146). Six samples were analyzed and MPH was found at concentrations of 3.4 μM to >100 μM. There has only been at least one MPH toxicity death reported in literature. In this case, the decedent had 331 MPH pills that were unaccounted for. MPH was found at 1100 ng/mL in peripheral blood and 980 ng/mL in central blood (135). The medical examiner ruled this an accidental death due to acute MPH intoxication. MPH continues to be a forensic concern as MPH is detected in a variety of settings. Many clinical studies analyze MPH in biological samples after controlled administration. Arvidsson et al. analyzed blood, breath and oral fluid samples after oral 20 mg MPH tablet in 12 subjects (139). Including the pre-dose sample, blood samples were collected at 18 time-points over 24 hours. The *l* enantiomer of MPH was detected in 5 out of 12 subject's plasma while *d*-MPH was detected in all samples for at least 15 hours. Both

enantiomers of RA were detected in all plasma samples (n=18/subject, first sample collection 0.5 hours post-administration) for every post-dose sample. In plasma, the concentrations of *d*- RA and *l*-RA were higher than the corresponding *d*-MPH and *l*-MPH concentrations, respectively (mean: 25-fold and 315-fold, respectively). In exhaled breath (n=14 samples/subject, first collection 0.5 hour post-administration), MPH was detected in 87% of samples while RA was only detected in 1%. In five subjects, MPH was detected in breath samples up to 24 hours after the dose though a consistent concentration pattern was not observed. MPH was detected in all oral fluid samples (n=10 samples/subject, first collection 1.5 hour post-administration) for up to 8 hours. (139). When analyzing RA, the plasma to oral fluid ratio was 32 with a higher concentration in plasma. To note, this same ratio was 1.8 for MPH. As with many drugs of abuse, impairing effects are likely if not taken as prescribed. When comparing forensic cases to clinical ones, there are similar concentrations reported. Of the postmortem cases described, MPH concentrations were often within the therapeutic range. Though MPH may be detected in forensic casework, it may or may not be found in concentrations that contribute to intoxication or toxicity. The only exception was the single MPH toxicity case in which MPH was at a concentration of 1100 ng/mL (135). Even if MPH does not contribute to cause of death, the abuse of this drug may lead to unwanted adverse effects.

Table 1.4. Antemortem and postmortem concentrations of MPH and RA reported in literature

Analyte	Case Type	N	Age	Sex	Concentration (ng/mL)[1]	Matrix	Other Analytes Detected	Case Notes	Reference
MPH RA	AM	5	10-17	4M, 1F	7.2-22 303-470	BL		Clinical study	(134)
MPH RA	AM	5	18-45	3M, 2F	6.2-13 193-484	BL		Clinical study	(134)
MPH RA	AM	10	20-35	9M, 1F	trace-22 55-2080	BL	Amphetamine, paracetamol, THC, diazepam/nordiazepam fluoxetine, tramadol	DUID	(134)
MPH RA	AM	7	14-57	3M, 4F	trace-23 60-3900	BL	Biperidene, carbamazepine, propiomazine, risperidone, sertraline, zolpidem, modafinial, duloxetine, enalapril, olanzapine	Therapeutic drug monitoring - prescription of Concerta® or Ritalin®	(134)
MPH RA	AM	12	22-42	6M, 6F	*l*-MPH in detected in 5 subjects, *d*-MPH detected in all RA detected in all samples	BL		20 mg dose of MPH	(139)
MPH	AM	1		F	2.04-15.72 (over 9.5 hours)	PL		17.5 mg dose of Ritalin (MPH)	(140)
MPH	AM	1		F	460-4020 nM	UR		25 mg dose of MPH	(146)
MPH	AM	6			3.4 uM to >100 uM	UR		Drivers who expressed drowsiness and hyperactivity	(146)
MPH RA	AM	5	10-17	4M, 1F	16-88 9.3-17	OF		Clinical study	(134)
MPH RA	AM	5	18-45	3M, 2F	18-49 6.4-22	OF		Clinical study	(134)
MPH RA	AM	111	6-12		3.5-61.3 2.6-47.0	OF		149 samples collected from children with ADHD	(150)
MPH	AM	12	22-42	6M, 6F	Detected in all OF samples	OF		20 mg dose of MPH	(139)
MPH	AM	19	21-34	10M, 9F	Initial: 7 Second peak: 9.3 (Ritalin) Initial: 3.4 Second peak: 5.9 (Concerta)	OF		20 mg dose of Ritalin and 10 mg dose Concerta	(154)

(continued)

Analyte	Case Type	N	Age	Sex	Concentration (ng/mL)[1]	Matrix	Other Analytes Detected	Case Notes	Reference
MPH RA	AM	12	22-42	6M, 6F	Detectable in 87% of samples Detectable in 1% of samples	Breath		20 mg dose of MPH	(139)
MPH RA	AM	7 3			150-10,400 pg/filter 35-360 pg/filter	Breath		80-400 mg dose of MPH	(155)
MPH	AM	1	26	F	1 pg/mg	Hair		Suspect was acting violently and charged with assault - claims drink was spiked with Ritalin	(152)
d-MPH l-MPH d-RA l-RA	PM	12	26-49	8M, 4F	5-58 ng/g ND-48 ng/g 24-782 ng/g 30-1174 ng/g	BL (femoral)	Benzodiazepines, methadone, morphine, codeine, pregabalin, oxycodone, methamphetamine, amphetamine, zopiclone, THC, ethanol, cocaine, anabolic steroids, GHB, heroin	No cause of death related to MPH	(156)
MPH RA	PM	10	25-49	8M, 2F	trace-95 15-974	BL (femoral)	Morphine, 6MAM, tamoxifen, THC, amphetamine, buprenorphine, diazepam, nordiazepam, norbuprenorphine, 7-amino-clonazepam, quetiapine, dihydropropiomazine, hydroxizine, mirtazapine, paracetamol, venlafaxine, citalopram, 7-amino nitrazepam, carbamazepine	Autopsy	(134)
MPH	PM	1	62	F	1100 980 3600 800 1 mg	BL (peripheral) BL (central) Liver Vitreous humor Stomach contents		Prescribed 10 mg tablets of MPH to be taken twice a day Prescription was filled 12 days prior to death 331 of 360 were missing	(135)

Abbreviations: methylphenidate (MPH), ritalinic acid (RA), blood (BL), plasma (PL), urine (UR), oral fluid (OF), antemortem (AM), postmortem (PM), male (M), female (F), driving under the influence of drugs (DUID), not detected (ND)
[1]units are in ng/mL unless otherwise specified

Alterative matrices – oral fluid

Oral fluid (OF) has emerged as an alternative biological matrix in both forensic and clinical settings. OF offers quick and non-invasive sample collection that can be conducted on-site. OF allows for reduced biohazard waste (compared to blood) and may reflect recent drug use (compared to urine) without the need for medically-trained or same-sex collectors. OF collection has been incorporated into drug treatment, workplace, pain management, and DUID programs (157). There are a few techniques for OF collection: passive drool, salivary stimulation, or collection devices. Collection devices usually contain a pad that absorbs a set volume of OF in a few minutes. A buffer is then used to stabilize the drugs (157). According to the Recommendations for Toxicological Investigations of Drug-Impaired Driving and Motor Vehicle Fatalities – 2017 Update, when investigating DUID cases, blood and oral fluid are the preferred specimens for collection (158). However, comprehensive analytical methods are needed for drug detection in oral fluid. There have been few LC-MS/MS methods that have analyzed MPH in OF. Mariotti et al. analyzed MPH (with fenproporex and diethylpropione) using LLE with a 2.5 ng/mL LOQ (149). Mulet et al. analyzed MPH and RA in OF with a LOQ of 0.5 ng/mL. This method was applied to 111 OF samples collected from children diagnosed with ADHD and MPH and RA were 3.5-61.3 and 2.6-47.0 ng/mL, respectively (150). Arvidsson et al. developed a method for MPH and RA (LOQ: 0.7 and 0.2 ng/mL, respectively) in OF using PP and applied it to 12 subjects who ingested a 20 mg MPH tablet (139). Lastly, MPH was analyzed in OF samples in two clinical studies between two age groups and MPH concentrations were 16-88 ng/mL (age: 10-17 years) and 18-49 ng/mL (age: 18-45 years) (134). As MPH is commonly prescribed and also abused, both

clinical and forensic laboratories should include such cognitive stimulants in their analytical scope of testing for OF.

Alternative instrumentation – supercritical fluid chromatography

With the ever-changing field of forensic toxicology, there needs to be alternative and novel ways to detect drugs of abuse that offer improvements in efficiency. Supercritical fluid chromatography (SFC) is a type of chromatography that utilizes a supercritical fluid as the mobile phase. A supercritical fluid is a substance at a temperature and pressure above its critical point. This supercritical fluid possesses characteristics of both a liquid and a gas (159). Due to the combination of these gas-like transfer properties and liquid-like solvation patterns, SFC proves to be promising instrument for chromatographic separation (160, 161). Carbon dioxide (CO_2) is the most common mobile phase used in SFC (159, 161). CO_2 has a low critical temperature and pressure but is non-toxic and easily affordable (160). Due to the polarity of CO_2, an organic modifier such as methanol (MeOH), ethanol or acetonitrile, is commonly added to the mobile phase to increase elution strength. Additives may also be used to improve peak shape for strongly polar compounds such as amines. The advantages of SFC when compared to LC are increased speed, better selectivity and higher efficiency (159). With the mobile phase being at a supercritical point, diffusion is higher than that of a liquid (used in LC) so SFC is faster than LC. Additionally, there is a lower viscosity of the mobile phase which improves SFC efficiency. SFC has been known to play a role in chiral separations due to the normal-phase HPLC environment that SFC has with a non-polar mobile phase. As compared to LC, SFC offers shortened analysis time, reduced solvent consumption, and improved retention time reproducibility (159). Also, SFC can

be coupled to GC and LC with other detectors such as Fourier transform infrared spectrometer or flame ionization detectors which allows for broad application (161).

MPH and EPH have been analyzed using SFC though the methods are limited. In a study by Desfontaine et al. MPH was analyzed in a study with 38 compounds (benzodiazepines, narcotics, stimulants, antidepressants, anesthetics, beta-blockers, antipsychotics and antihistamines) that was comparing SFC columns for the best peak shape. The SFC mobile phase was CO_2/MeOH with a 10 mM ammonium formate (AMF) additive (162). In another study by Desfontaine et al. the matrix effects of plasma and urine were tested on both SFC and LC for a variety of drugs, including MPH. Both matrices underwent a clean-up procedure (dilution for urine and protein precipitation for plasma) and a selective sample preparation (solid-phase extraction). The SFC mobile phase was CO_2/10 mM AMF with 98:2 MeOH:H_20. This study concluded that matrix effects are reduced with SFC compared to LC, especially when SPE is used (163). Novakova et al. assessed MPH and 110 other doping agents and found that UHPSFC-MS/MS was more sensitive for 32% of these agents when compared to UHPLC-MS/MS (164). Perrenoud et al. tested 92 pharmaceutical drugs, including MPH with an SFC mobile phase of CO_2/MeOH. This study found that addition of 20 mM ammonium hydroxide as an additive can help improve peak shape of some analytes (165). Lastly, EPH was analyzed with 40 novel psychoactive substances (NPS) (pyrovalerones, benzofurans, phenidates, phenidines) to test which additive pairs best with CO_2 for enantiomeric separation. In this study, 88% of NPS tested were enantio-separated with SFC as compared to only 36% with LC. Similar to previous studies, they indicated that MeOH was best paired with CO_2 in the mobile phase to assist with enantioseparation

(166). As chiral derivatization for GC or chiral columns for LC are costly and time consuming, SFC may be a viable technique for chiral separation of MPH and EPH enantiomers. SFC offers improved sensitivity, peak shape, and matrix effects.

Stability

Stability of analytes is important to study and understand to ensure proper analysis of samples. If analytes are not stable in various matrices under certain conditions, inaccurate quantification values may be reported. Analyte degradation can occur so samples need to be properly stored during transportation and before analysis to ensure accurate reporting. Also, time before analysis plays a crucial role in the stability of analytes. Stability studies can give valuable data for various storage conditions and how long analytes remain stable in those conditions over a specified amount of time. There have been stability studies analyzing MPH. Thomsen analyzed MPH for one month in blood at ambient temperature, 4ºC, -20ºC and -80ºC. The enantiomers of MPH remained stable in all conditions with the exception of ambient temperature (127). Secilir et al. conducted a stability study in plasma and saliva and found that MPH was stable at -20ºC for one month but not at 5ºC for 48 hours (138). Ramos et al. found that *d*-MPH is not stable at ambient temperature for 24 hours while *l*-MPH remained stable for >24 hours (136). Stability has been conducted with various freeze-thaw cycles and MPH has remained stable (62, 126, 132, 137) in them all. There have been few studies that have analyzed MPH over a long period of time. Thomsen et al. and Leis et al. analyzed MPH at -20ºC for 17 and 30 days, respectively. MPH remained stable in both studies (127, 137). Zhang et al. analyzed MPH at this same condition for six months and found that

MPH remained stable during this time (62). Other than the study by Zhang et al. there is a lack of long-term stability studies for MPH.

Ethylphenidate

Prevalence

Ethylphenidate was first synthesized as an internal standard when analyzing MPH PK (129) (Figure 1.4). In more recent years, it has emerged as a NPS under the street name “Nopaine”(130). NPS, or “legal highs,” are drugs sold as “research chemicals” and typically labeled “not for human consumption” (131, 132). There has been an alarming increase in NPS use over the last decade (133). Though identified over 50 years ago, EPH did not appear on a drug forum until 2010 (134) and was first reported to the EMCDDA Early Warning System in 2011 (135). EPH was first controlled in 2012-2013 by some European states and in 2015 under a Temporary Class Drug Order in the United Kingdom (136-139). EPH is not controlled in the United States though it could be considered an analog of MPH which would make it a Schedule II substance. Though EPH is metabolized from MPH in the presence of alcohol, there have been reports on internet forums that EPH is being abused as a standalone compound for its stimulant effects, making it of importance on the forensic radar.

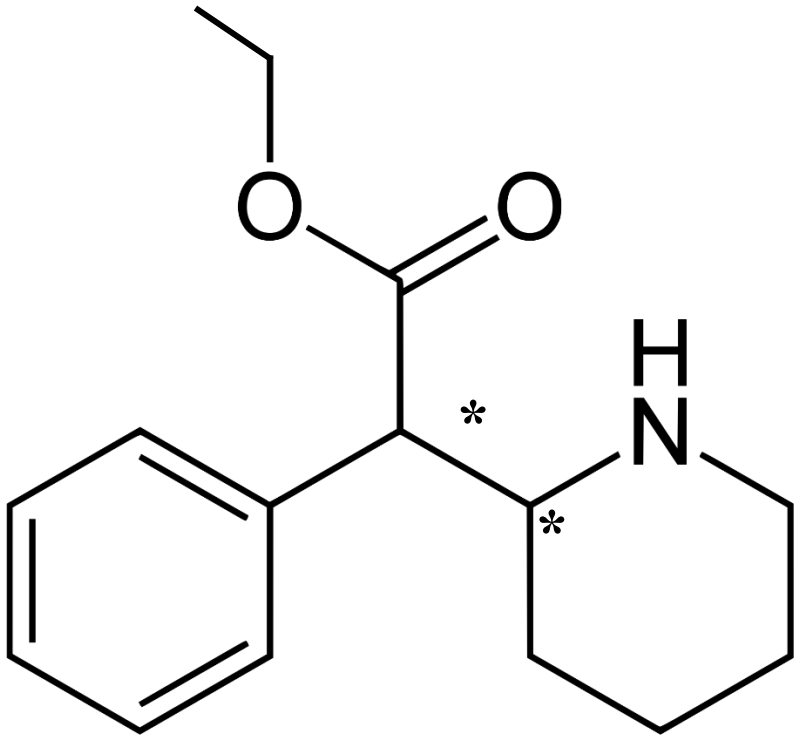

*Figure 1.4. Structure of ethylphenidate (chiral centers indicated by *)*

Between August 2010 and February 2015, there were 198 user reports on EPH internet forums and social media sites with (140). Ho et al. found that there were 83 internet sites (based out the UK) that sold illicit EPH with at least one other NPS (140). This demonstrates that EPH can no longer solely be considered a biomarker for MPH/alcohol use as it is being abused as an emerging designer drug. Internet sites were selling EPH as powders, crystals, or pellets with a maximum dose of 10 kg, 5 kg, and 50 mg, respectively. The recommended dose is 30 mg. The main route of administration reported was insufflation. EPH user reports indicate co-administration with many other substances including benzodiazepines, alcohol, other stimulants, cannabis, other sedatives or hypnotics, and GHB. Like with other stimulants, EPH users often self-medicate with benzodiazepines to reduce undesirable effects during crash phase (141). The most reported effect was euphoria followed by elation (140). When assessing use of EPH for

academic performance, some reports stated it was a good study aid (142) while others say the euphoria was distracting (143). Users who reported EPH use as a study aid stated it was similar to MPH (142, 144, 145). Among the 198 forums, there were 688 reports of unwanted effects with the top three being nasal pain, anxiety, and palpitations. After effects included the desire to use again and lethargy (140). Soussan et al. found 44 user reports of EPH use from March 2011 to March 2014. EPH was reported to be used for recreational use, cognitive enhancement and increase in social interaction experiences. Similar to Ho et al., many users reported nasal pain after insufflation, anxiety, increased blood rate, and profuse sweating. Half of the users in the Soussan et al. study also reported the desire to reuse (146). Though there were mixed reviews on the strength of the effects, users reported EPH effects similar to those of cocaine, amphetamine, mephedrone, pentedrone, 3,4-methylenedioxymethamphetamine and methylenedioxypyrovalerone (142-145). Though there are few analytical studies encompassing EPH, it is considered a NPS with high potential for recreational abuse. As such, analytical methods are needed to detect and quantify EPH as it is not just a metabolite of MPH but a designer drug that can be ingested alone.

Pharmacology

EPH, or (2R:2'R,2S:2'S)-α-phenyl-2-piperidineacetic acid ethyl ester is an active metabolite of MPH produced *in vivo* in the presence of alcohol (14) (147-149). The central nervous activity of EPH was first reported in 1961 and it was discovered that, in a mouse model, EPH was 80% more potent than MPH (150). When comparing to MPH, EPH has displayed a 16-fold higher affinity for DAT than NAT which increases the potential for abuse and addiction (151, 152). Due to the similar structure of MPH, EPH

has 2 chiral centers giving rise to 4 stereoisomers. Like MPH, all catecholaminergic activity is due to *d*-EPH (14). EPH may serve as a biomarker for evidence of concomitant MPH and ethanol use (121, 125, 149, 153). Since EPH has been reported to have more selective neurochemical actions than MPH, there is potential for EPH to act as a therapeutic agent for ADHD by more selectively targeting DAT (83, 106, 107).

Pharmacokinetics

In vivo, EPH is formed from MPH in the presence of alcohol (192, 193). Illicit MPH use usually involves the consumption of alcohol (88, 116, 194) leading to the production of EPH. EPH has similar effects as MPH with increased concentration and decreased inattentiveness. Physiologically, EPH increases heart rate, blood pressure and can lead to insomnia, sweating and loss of appetite (194). Given their structural similarity, the V_d of EPH might be expected to be similar to MPH, though little data are published to support this. Like RA, this metabolism is catalyzed enantioselectivity by the CES1A1 enzyme in the liver via transesterification (83, 188, 195, 196, 197). In the presence of alcohol, CES1A1 metabolism slows down which increases *d*-MPH in plasma, producing the euphoric effects that humans feel. Therefore, the effects from EPH do not happen due to EPH itself, but due to an increase in parent drug concentrations (83, 114, 198, 199). This pathway is like that of cocaine. In the presence of alcohol, cocaine is metabolized to cocaethylene but ethanol increases the concentration of parent cocaine which increases the stimulant effects (200). Due to the common co-abuse of MPH and ethanol, it is relevant to study EPH in forensic cases.

Methods

There are few analytical methods for detection and quantification of EPH in human matrices. These methods are summarized in Table 1.5. Of those methods that have been developed, there are few that are enantioselective. Zhu et al. developed a chiral method for the separation of EPH enantiomers in plasma using LLE (126). The enantiomers were separated using an Astec Chirobitoic V2 chiral column on a LC-MS/MS with 0.025 ng/mL LOQ. EPH has been studied in other matrices such as serum (200, 201), hair (201, 202), blood (203) and urine (201). More recently, screening techniques targeting NPS and designer stimulants that include EPH were developed for blood (204, 205) using LC-MS/MS. In the screen by Giorgetti et al., EPH LOQ was 0.51 ng/mL (204). With EPH emerging as an NPS as well as a biomarker for MPH use in forensic and clinical settings, methods are needed to detect and quantify this analyte. In an effort to understand pharmacological properties of the enantiomers and to assist with forensic toxicological data interpretation, there remains a need for chiral separation of EPH enantiomers in biological matrices.

Table 1.5. Analytical methods for quantification of EPH in human and biological matrices as reported in literature

Matrix	Analyte	Chiral Separation Mechanism	ISTD	Extraction	Instrument	Calibration Range (ng/mL)[1]	LOQ (ng/mL)[1]	Other Analytes	Reference
BL	EPH			PP	LC-MS/MS	1-250			(203)
PL	**d,l* -EPH	Chiral column	*d,l* -MPH-d3	LLE	LC-MS/MS	0.025-25	0.02	*d,l* -MPH	(126)
PL (rat)	**d,l* -EPH	Chiral column	MPH-d9	LLE	LC-MS/MS	1-500	1	*d,l* -MPH *d,l* -RA	(142)
PL	*d,l* -EPH		MPH-d3	SPE	LC-MS/MS	0.05-5	<0.05		
Serum	EPH		MPH-d3	SPE	LC-MS/MS	5-1000	5		(200)
Serum	EPH			SPE PP	GC-MS LC-MS/MS		10 5	5-APB, 5-MAPB, 5-EAPB, RA	(201)
UR	EPH			SPE PP	GC-MS LC-MS/MS		10 5		
Hair	EPH			Wash SPE	LC-MS/MS GC-MS		5 0.2		
Hair	EPH				LC-MS/MS	10-500 pg/mg	10.3 pg/mg	97 other NPS	(202)

Abbreviations: methylphenidate (MPH), ritalinic acid (RA), ethylphenidate (EPH), blood (BL), plasma (PL), urine (UR), liquid chromatography-tandem mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC-MS), protein precipitation (PP), liquid-liquid extraction (LLE) solid-phase extraction (SPE)

*chiral separation

[1]units are in ng/mL unless otherwise specified

Cases

There have been few reports cases associated with ethylphenidate intake. Of those that have been reported, most of them are postmortem cases in which EPH was detected. In an effort to study EPH formation, Markowitz et al. administered two 10 mg MPH tablets to six volunteers followed by ingestion of ethanol (196). EPH was detectable in plasma of all subjects at a mean concentration of 0.43 ng/mL, establishing EPH as a metabolite of MPH after ethanol ingestion. In an antemortem case of EPH toxicity, EPH was found in concentrations of 0.24 and 0.98 ng/mL in serum and urine, respectively, after ingesting 500 mg of EPH (206). From a postmortem standpoint, Maskell et al. reviewed seven fatalities associated with EPH (200). Of the seven cases, six blood samples were positive for other drugs. In the single EPH toxicity case, EPH was 2180 ng/mL in blood (200). Krueger et al. had two cases in which EPH was detected in autopsy cases following a routine screen of the organs and biological fluids. EPH was found in combination with other drugs in both cases (203). Lastly, Parks et al. conducted a study of postmortem cases in Scotland from 2013-2014 and found 19 cases with detectable EPH at concentrations of 8-2000 ng/mL (207). In every case, other drugs of abuse were detected with EPH. Of those 19, the cause of death in five of those was directly related to EPH toxicity. These data are summarized in Table 1.6. In postmortem samples, EPH was present at higher concentrations compared to antemortem samples. In the postmortem EPH toxicity case in which EPH was the only drug suspected, the concentration was 2180 ng/mL compared to the antemortem single EPH case at 0.24 ng/mL in plasma. Additionally, EPH concentrations in antemortem cases were higher than when detected with other drugs (range: 30-1370 ng/mL). These cases indicate that

EPH toxicity can be fatal, especially when taken in combination with other drugs of abuse. This makes it necessary to have analytical methods to quantify EPH in various matrices.

Table 1.6. Antemortem and postmortem concentrations of EPH reported in literature

Analyte	Case Type	N	Age	Sex	Concentration (ng/mL)[1]	Matrix	Other Analytes	Notes	Reference
EPH	AM	1	21	M	0.24 0.98	Serum UR		Reported using 500 mg of EPH over several hours	(206)
EPH	AM	6	24-32	3M, 3F	0.43 (mean C_{max})	PL		10 mg MPH (x2) + 0.6 g/kg ethanol 30 minutes later	(196)
EPH	PM	1	38	M	23	BL (femoral)	Fentanyl, norfentayl, pregabalin, RA	Routine screen	(203)
EPH	PM	19	20-54	14M, 5F	8-2000	BL (femoral)	Benzodiazepines, opiates, methadone	Postmortem autopsy findings; EPH was cause of death in 5 cases	(207)
EPH	PM	7	23-49		2180	BL (femoral)		COD: EPH toxicity	(200)
					1370		Benzoylecgonine, sertraline, diphenhydramine	COD: hanging	(200)
					870		Dothiepin, methiopropamine, ethanol	COD: hanging	(200)
					110		Methadone, EDDP, zopiclone, sertraline, aripiprazole, dehydroaripiprazole, 2-aminoindane, ethanol	COD: methadone and 2 amino toxicity	(200)
					140		Morphine, codeine, ketamine, cocaine, benzoylecgonine, venlafaxine, o-desmethylvenlafaxine	COD: heroin toxicity	(200)
					30		Methiopropamine, 5APB/6APB	COD: mixed drug toxicity	(200)
					110		Diazepam, nordiazepam, temazepam, oxazepam, morphine, codeine	COD: multiple drug toxicity	(200)
EPH	PM	1	32	M	110	BL (femoral)	Methadone, EDDP, morphine, fentanyl, MPH, RA	Routine screen	(203)
					180	Liver			
					130	Pericardial fluid			
					980	UR	Methadone, EDDP, morphine, fentanyl, MPH, RA		
					20	Stomach contents	Methadone, EDDP, morphine, MPH		

Abbreviations: ethylphenidate (EPH), blood (BL), plasma (PL), urine (UR), antemortem (AM), postmortem (PM), male (M), female (F), cause of death (COD)

[1]units are in ng/mL unless otherwise specified

Stability

There are few methods that have analyzed stability of EPH and its enantiomers. Zhang et al. analyzed EPH in rat plasma at ambient temperature (4 and 24 hours), -80ºC (30 days), and for three freeze-thaw cycles (62). EPH was considered stable under these conditions. Additionally, Zhu et al. conducted a stability study in human plasma for EPH. The enantiomers of EPH remained stable at room temperature (4 hours), in the autosampler after processing (4ºC, 24 hours) and for three freeze-thaw cycles (126). To the authors knowledge there are no long-term EPH stability studies. As previously mentioned, stability studies are necessary to understand storage, handling conditions, and proper analysis time to ensure accurate quantification values.

Statement of the Problem

Due to the increase in prescribed stimulant medications among young adults, there is a potential for abuse among this population. As the purpose of these stimulants is to speed up brain activity, there is an increased risk that they may be misused or abused in an academic setting. As previous studies show, there is a likelihood that these prescriptions may be illicitly diverted. Due to the potential for abuse, it is important to monitor cognitive stimulants in clinical and forensic settings. This study aims to address analytical gaps in the literature by developing enantioselective separation techniques for analysis of MPH and its metabolites in various biological samples. As EPH is produced from MPH in the presence of alcohol, which is commonly co-abused with MPH, this analyte is of importance as well. Additionally, as EPH is emerging as a NPS, it is important to monitor this analyte as it may be used illicitly and its presence may not necessarily indicate MPH intake. This study seeks to provide validated analytical

methods that can be used in clinical and forensic laboratories to detect and quantify MPH, EPH, RA, and other cognitive stimulants in blood and oral fluid using liquid chromatography-tandem mass spectrometry. Additionally, this study addresses pressing issues of MPH stability in an effort to understand analyte degradation and to ensure proper storage and handling of forensic samples for accurate quantification and data interpretation.

References

1. Berger, I. (2011) Diagnosis of attention deficit hyperactivity disorder: much ado about something. Israel Medical Associaiton Journal, 13, 571-4.
2. Fayyad, J., Sampson, N.A., Hwang, I., Adamowski, T., Aguilar-Gaxiola, S., Al-Hamzawi, A., et al. (2017) The descriptive epidemiology of DSM-IV Adult ADHD in the World Health Organization World Mental Health Surveys. ADHD Attention Deficit and Hyperactivity Disorders, 9, 47-65.
3. Kooij, J.J.S., Bijlenga, D., Salerno, L., Jaeschke, R., Bitter, I., Balázs, J., et al. (2019) Updated European Consensus Statement on diagnosis and treatment of adult ADHD. European Psychiatry, 56, 14-34.
4. Polanczyk, G.V., Salum, G.A., Sugaya, L.S., Caye, A. and Rohde, L.A. (2015) Annual research review: A meta-analysis of the worldwide prevalence of mental disorders in children and adolescents. Journal of Child Psychology and Psychiatry, 56, 345-365.
5. Reed, G.M., First, M.B., Kogan, C.S., Hyman, S.E., Gureje, O., Gaebel, W., et al. (2019) Innovations and changes in the ICD-11 classification of mental, behavioural and neurodevelopmental disorders. World Psychiatry, 18, 3-19.
6. Volkow, N.D. and Swanson, J.M. (2013) Adult attention deficit–hyperactivity disorder. New England Journal of Medicine, 369, 1935-1944.
7. Issues., A.W.G.o.Q. (2007) Practice parameter for the assessment and treatment of children and adolescents with attention-deficit/hyperactivity disorder. Journal of the American Academy of Child and Adolescent Psychiatry, 46, 894-921.

8. Jaeschke, R.R., Sujkowska, E. and Sowa-Kućma, M. (2021) Methylphenidate for attention-deficit/hyperactivity disorder in adults: a narrative review. Psychopharmacology, 238, 2667-2691.
9. Childress, A.C. and Berry, S.A. (2012) Pharmacotherapy of attention-deficit hyperactivity disorder in adolescents. Drugs, 72, 309-325.
10. Trent, S. and Davies, W. (2012) The influence of sex-linked genetic mechanisms on attention and impulsivity. Biological Psychology, 89, 1-13.
11. Breda, V., Rohde, L.A., Menezes, A.M.B., Anselmi, L., Caye, A., Rovaris, D.L., et al. (2021) The neurodevelopmental nature of attention-deficit hyperactivity disorder in adults. British Journal of Psychiatry, 218, 43-50.
12. Demontis, D., Walters, R.K., Martin, J., Mattheisen, M., Als, T.D., Agerbo, E., et al. (2019) Discovery of the first genome-wide significant risk loci for attention deficit/hyperactivity disorder. National Genetics, 51, 63-75.
13. Faraone, S.V., Banaschewski, T., Coghill, D., Zheng, Y., Biederman, J., Bellgrove, M.A., et al. (2021) The World Federation of ADHD International Consensus Statement: 208 Evidence-based conclusions about the disorder. Neuroscience & Biobehavioral Reviews, 128, 789-818.
14. Biederman, J. and Spencer, T. (2002) Methylphenidate in treatment of adults with Attention-Deficit/Hyperactivity Disorder. Journal of Attention Disorders, 6 Suppl 1, S101-7.
15. Maul, J. and Advokat, C. (2013) Stimulant medications for attention-deficit/hyperactivity disorder (ADHD) improve memory of emotional stimuli in

ADHD-diagnosed college students. Pharmacology Biochemistry and Behavior, 105, 58-62.

16. Modesto-Lowe, V., Meyer, A. and Soovajian, V. (2012) A clinican's guide to adult attention-deficit hyperactivity disorder. Conneicut Medicine, 76.
17. Cortese, S. (2012) The neurobiology and genetics of attention-deficit/hyperactivity disorder (ADHD): what every clinician should know. European Journal of Paediatrics, 16, 422-433.
18. Biederman, J., Faraone, S.V., Spencer, T.J., Mick, E., Monuteaux, M.C. and Aleardi, M. (2006) Functional impairments in adults with self-reports of diagnosed ADHD: A controlled study of 1001 adults in the community. Journal of Clinical Psychiatry, 67, 524-540.
19. Sharma, A. and Couture, J. (2013) A Review of the Pathophysiology, Etiology, and Treatment of Attention-Deficit Hyperactivity Disorder (ADHD). Annals of Pharmacotherapy, 48, 209-225.
20. Kehoe, W.A. (2001) Treatment of attention deficit hyperactivity disorder in children. Annals of Pharmacotherapy, 35, 1130-4.
21. Tripp, G. and Wickens, J.R. (2009) Neurobiology of ADHD. Neuropharmacology, 57, 579-589.
22. Fusar-Poli, P., Rubia, K., Rossi, G., Sartori, G. and Balottin, U. (2012) Striatal dopamine transporter alterations in ADHD: pathophysiology or adaptation to psychostimulants? A meta-analysis. American Journal of Psychiatry, 169, 264-272.

23. Arnsten, A.F. and Pliszka, S.R. (2011) Catecholamine influences on prefrontal cortical function: relevance to treatment of attention deficit/hyperactivity disorder and related disorders. Pharmacology Biochemistry and Behavior, 99, 211-216.

24. Kesner, R.P. and Churchwell, J.C. (2011) An analysis of rat prefrontal cortex in mediating executive function. Neurobiology of Learning and Memory, 96, 417-431.

25. Arnsten, A.F. (2007) Catecholamine and second messenger influences on prefrontal cortical networks of "representational knowledge": a rational bridge between genetics and the symptoms of mental illness. Cerebral Cortex, 17, i6-i15.

26. Arnsten, A.F., Paspalas, C.D., Gamo, N.J., Yang, Y. and Wang, M. (2010) Dynamic network connectivity: a new form of neuroplasticity. Trends in Cognitive Science, 14, 365-375.

27. Pliszka, S.R. (2005) The neuropsychopharmacology of attention-deficit/hyperactivity disorder. Biological Psychiatry, 57, 1385-1390.

28. Robbins, T.W. (2003) Dopamine and cognition. Curr Opin Neurol, 16, S1-S2.

29. Janowsky, A., Schweri, M.M., Berger, P., Long, R., Skolnick, P. and Paul, S.M. (1985) The effects of surgical and chemical lesions on striatal [3H] threo-(±)-methylphenidate binding: correlation with [3H] dopamine uptake. European Journal of Pharmacology, 108, 187-191.

30. Schweri, M.M., Skolnick, P., Rafferty, M.F., Rice, K.C., Janowsky, A.J. and Paul, S.M. (1985) [3H]Threo-(±)-Methylphenidate Binding to 3,4-Dihydroxyphenylethylamine Uptake Sites in Corpus Striatum: Correlation with the Stimulant Properties of Ritalinic Acid Esters. Journal of Neurochemistry, 45, 1062-1070.

31. Hurd, Y.L. and Ungerstedt, U. (1989) In vivo neurochemical profile of dopamine uptake inhibitors and releasers in rat caudate-putamen. European Journal of Pharmacology, 166, 251-260.

32. Butcher, S., Liptrot, J. and Aburthnott, G. (1991) Characterisation of methylphenidate and nomifensine induced dopamine release in rat striatum using in vivo brain microdialysis. Neuroscience, 122, 245-248.

33. Wall, S.C., Gu, H. and Rudnick, G. (1995) Biogenic amine flux mediated by cloned transporters stably expressed in cultured cell lines: amphetamine specificity for inhibition and efflux. Molecular Pharmacology, 47, 544-550.

34. Gizer, I.R., Ficks, C. and Waldman, I.D. (2009) Candidate gene studies of ADHD: a meta-analytic review. Journal of Human Genetics, 126, 51-90.

35. Group, M.C. (2004) National Institute of Mental Health Multimodal Treatment Study of ADHD follow-up: changes in effectiveness and growth after the end of treatment. Pediatrics, 113, 762-769.

36. Spencer, T.J., Wilens, T.E., Biederman, J., Weisler, R.H., Read, S.C. and Pratt, R. (2006) Efficacy and safety of mixed amphetamine salts extended release (Adderall XR) in the management of attention-deficit/hyperactivity disorder in adolescent patients: a 4-week, randomized, double-blind, placebo-controlled, parallel-group study. Clinical Therapeutics, 28, 266-279.

37. Wilens, T.E., McBurnett, K., Bukstein, O., McGough, J., Greenhill, L., Lerner, M., et al. (2006) Multisite controlled study of OROS methylphenidate in the treatment of adolescents with attention-deficit/hyperactivity disorder. Archives of Pediatrics and Adolescent Medicine, 160, 82-90.

38. Pliszka, S.R. (2007) Pharmacologic treatment of attention-deficit/hyperactivity disorder: efficacy, safety and mechanisms of action. Neuropsychology Review, 17, 61-72.

39. Dopheide, J.A. and Pliszka, S.R. (2009) Attention-deficit-hyperactivity disorder: an update. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 29, 656-679.

40. Faraone, S.V. (2009) Using meta-analysis to compare the efficacy of medications for attention-deficit/hyperactivity disorder in youths. Pharmacy and Therapeutics, 34, 678.

41. Biederman, J., Boellner, S.W., Childress, A., Lopez, F.A., Krishnan, S. and Zhang, Y. (2007) Lisdexamfetamine dimesylate and mixed amphetamine salts extended-release in children with ADHD: a double-blind, placebo-controlled, crossover analog classroom study. Biological Psychiatry, 62, 970-6.

42. Stevens, J.R., George, R.A., Fusillo, S., Stern, T.A. and Wilens, T.E. (2010) Plasma methylphenidate concentrations in youths treated with high-dose osmotic release oral system formulation. Journal of Child and Adolescent Psychopharmacology, 20, 49-54.

43. Seiden, L.S., Sabol, K.E. and Ricaurte, G.A. (1993) Amphetamine: effects on catecholamine systems and behavior. Annual Review of Pharmacology and Toxicology, 33, 639-676.

44. Levi, G. and Raiteri, M. (1993) Carrier-mediated release of neurotransmitters. Trends in Neurosciences, 16, 415-419.

45. Volkow, N.D., Ding, Y.-S., Fowler, J.S., Wang, G.-J., Logan, J., Gatley, J.S., et al. (1995) Is methylphenidate like cocaine?: Studies on their pharmacokinetics and distribution in the human brain. Archives of General Psychiatry, 52, 456-463.

46. Nutt, D., King, L.A., Saulsbury, W. and Blakemore, C. (2007) Development of a rational scale to assess the harm of drugs of potential misuse. The Lancet, 369, 1047-1053.

47. Smith, M.E. and Farah, M.J. (2011) Are prescription stimulants "smart pills"? The epidemiology and cognitive neuroscience of prescription stimulant use by normal healthy individuals. Psychological Bulletin, 137, 717.

48. Poulton, A. (2005) Growth on stimulant medication; clarifying the confusion: a review. Archives of Disease in Childhood, 90, 801-806.

49. Faraone, S.V., Biederman, J., Monuteaux, M. and Spencer, T. (2005) Long-term effects of extended-release mixed amphetamine salts treatment of attention-deficit/hyperactivity disorder on growth. Journal of Child and Adolescent Psychopharmacology, 15, 191-202.

50. Swanson, J., Greenhill, L., Wigal, T., Kollins, S., Stehli, A., Davies, M., et al. (2006) Stimulant-related reductions of growth rates in the PATS. Journal of the American Academy of Child and Adolescent Psychiatry, 45, 1304-1313.

51. Berridge, C.W., Devilbiss, D.M., Andrzejewski, M.E., Arnsten, A.F., Kelley, A.E., Schmeichel, B., et al. (2006) Methylphenidate preferentially increases catecholamine neurotransmission within the prefrontal cortex at low doses that enhance cognitive function. Biological Psychiatry, 60, 1111-1120.

52. Biederman, J., Wilens, T., Mick, E., Spencer, T. and Faraone, S.V. (1999) Pharmacotherapy of attention-deficit/hyperactivity disorder reduces risk for substance use disorder. Pediatrics, 104, e20-e20.

53. Biederman, J. (2003) Pharmacotherapy for attention-deficit/hyperactivity disorder (ADHD) decreases the risk for substance abuse: findings from a longitudinal follow-up of youths with and without ADHD. Journal of Clinical Psychiatry, 64, 3-8.

54. Mannuzza, S., Klein, R.G., Truong, N.L., Moulton III, P.D., John L, Roizen, E.R., Howell, K.H., et al. (2008) Age of methylphenidate treatment initiation in children with ADHD and later substance abuse: prospective follow-up into adulthood. American Journal of Psychiatry, 165, 604-609.

55. Biederman, J., Monuteaux, M.C., Spencer, T., Wilens, T.E. and Faraone, S.V. (2009) Do stimulants protect against psychiatric disorders in youth with ADHD? A 10-year follow-up study. Pediatrics, 124, 71-78.

56. Connor, D.F., Glatt, S.J., Lopez, I.D., Jackson, D. and Melloni Jr, R.H. (2002) Psychopharmacology and aggression. I: A meta-analysis of stimulant effects on overt/covert aggression–related behaviors in ADHD. Journal of the American Academy of Child and Adolescent Psychiatry, 41, 253-261.

57. Advokat, C. (2009) What exactly are the benefits of stimulants for ADHD? Journal of Attention Disorders, 12, 495-498.

58. Advokat, C. (2010) What are the cognitive effects of stimulant medications? Emphasis on adults with attention-deficit/hyperactivity disorder (ADHD). Neuroscience and Biobehavioral Reviews, 34, 1256-1266.

59. Gamo, N.J., Wang, M. and Arnsten, A.F. (2010) Methylphenidate and atomoxetine enhance prefrontal function through α2-adrenergic and dopamine D1 receptors. Journal of the American Academy of Child and Adolescent Psychiatry, 49, 1011-1023.

60. Volkow, N.D., Wang, G.-J., Fowler, J.S., Telang, F., Maynard, L., Logan, J., et al. (2004) Evidence that methylphenidate enhances the saliency of a mathematical task by increasing dopamine in the human brain. American Journal of Psychiatry, 161, 1173-1180.

61. Volkow, N.D., Wang, G.-J., Fowler, J.S., Gatley, S.J., Logan, J., Ding, Y.-S., et al. (1998) Dopamine transporter occupancies in the human brain induced by therapeutic doses of oral methylphenidate. American Journal of Psychiatry, 155, 1325-1331.

62. Zhang, J., Deng, Y., Fang, J. and McKay, G. (2003) Enantioselective analysis of ritalinic acids in biological samples by using a protein-based chiral stationary phase. Pharmaceutical Research, 20, 1881-1884.

63. (2012) Adderall (dextroamphetamine saccharate, amphetamine aspartate, dextroamphetamine sulfate and amphetamine sulfate tablet) [product information]. In Pharmaceuticals, T. (ed.) North Wales, PA.

64. Advokat, C. (2007) Literature review: Update on amphetamine neurotoxicity and its relevance to the treatment of ADHD. Journal of Attention Disorders, 11, 8-16.

65. Spencer, T., Biederman, J. and Wilens, T. (2004) Nonstimulant treatment of adult attention-deficit/hyperactivity disorder. The Psychiatric Clinics of North America, 27, 373-383.

66. Waxmonsky, J.G. (2005) Nonstimulant therapies for attention-deficit hyperactivity disorder (ADHD) in children and adults. Essential Psychopharmacology, 6, 262-276.

67. Faraone, S.V. and Buitelaar, J. (2010) Comparing the efficacy of stimulants for ADHD in children and adolescents using meta-analysis. European Child and Adolescent Psychiatry, 19, 353-364.

68. Hazell, P.L., Kohn, M.R., Dickson, R., Walton, R.J., Granger, R.E. and van Wyk, G.W. (2011) Core ADHD symptom improvement with atomoxetine versus methylphenidate: a direct comparison meta-analysis. Journal of Attention Disorders, 15, 674-683.

69. Greenhill, L.L., Findling, R.L. and Swanson, J.M. (2002) A double-blind, placebo-controlled study of modified-release methylphenidate in children with attention-deficit/hyperactivity disorder. Pediatrics, 109, e39-e39.

70. Greenhill, L.L., Pliszka, S. and Dulcan, M.K. (2002) Practice parameter for the use of stimulant medications in the treatment of children, adolescents, and adults. Journal of the American Academy of Child & Adolescent Psychiatry, 41, 26S-49S.

71. Wernicke, J. and Kratochvil, C.J. (2002) Safety profile of atomoxetine in the treatment of children and adolescents with ADHD. Journal of Clinical Psychiatry, 63, 50-55.

72. Nelson, J.M. and Liebel, S.W. (2018) Anxiety and depression among college students with attention-deficit/hyperactivity disorder (ADHD): Cross-informant, sex, and subtype differences. Journal of American College Health, 66, 123-132.

73. Barkley, R.A. and Cunningham, C.E. (1978) Do stimulant drugs improve the academic performance of hyperkinetic children? A review of outcome studies. Clinical Pediatrics, 17, 85-92.

74. Cantwell, D.P. and Satterfield, J.H. (1978) The prevalence of academic underachievement in hyperactive children. Journal of Pediatric Psychology, 3, 168-171.

75. Carlson, C.L. and Bunner, M.R. (1993) Effects of methylphenidate on the academic performance of children with attention-deficit hyperactivity disorder and learning disabilities. School Psychology Review, 22, 184-198.

76. Gadow, K.D. (1983) Effects of stimulant drugs on academic performance in hyperactive and learning disabled children. Journal of Learning Disabilities, 16, 290-299.

77. Gualtieri, C.T. and Johnson, L.G. (2008) Medications do not necessarily normalize cognition in ADHD patients. Journal of Attention Disorders, 11, 459-469.

78. Loe, I.M. and Feldman, H.M. (2007) Academic and educational outcomes of children with ADHD. Journal of Pediatric Psychology, 32, 643-654.

79. Swanson, J.M., Cantwell, D., Lerner, M., McBurnett, K. and Hanna, G. (1991) Effects of stimulant medication on learning in children with ADHD. Journal of Learning Disabilities, 24, 219-230.

80. Bolea-Alamanac, B., Nutt, D., Adamou, M., Asherson, P., Bazire, S., Coghill, D., et al. (2014) Evidence-based guidelines for the pharmacological management of attention deficit hyperactivity disorder: Update on recommendations from the British Association for Psychopharmacology. Journal of Psychopharmacology, 28.

81. Bolea-Alamanac, B.M., Green, A., Verma, G., Maxwell, P. and Davies, S.J.C. (2014) Methylphenidate use in pregnancy and lactation: a systematic review of evidence. British Journal of Clinical Pharmacology, 77, 96-101.

82. Patrick, K.S., González, M.A., Straughn, A.B. and Markowitz, J.S. (2005) New methylphenidate formulations for the treatment of attention-deficit/hyperactivity disorder. Expert Opinion on Drug Delivery, 2, 121-143.

83. Antshel, K.M., Hargrave, T.M., Simonescu, M., Kaul, P., Hendricks, K. and Faraone, S.V. (2011) Advances in understanding and treating ADHD. BMC Medicine, 9, 72.

84. Raman, S.R., Man, K.K., Bahmanyar, S., Berard, A., Bilder, S., Boukhris, T., et al. (2018) Trends in attention-deficit hyperactivity disorder medication use: a retrospective observational study using population-based databases. The Lancet Psychiatry, 5, 824-835.

85. Ross, M.M., Arria, A.M., Brown, J.P., Mullins, C.D., Schiffman, J., Simoni-Wastila, L., et al. (2018) College students' perceived benefit-to-risk tradeoffs for nonmedical use of prescription stimulants: Implications for intervention designs. Addictive Behaviors, 79, 45-51.

86. Schulenberg, J., Johnston, L., O'Malley, P., Bachman, J., Miech, R. and Patrick, M. (2017) College Students & Adults Ages 19-55. In National Survey Results on Drug Use 1975-2017, Monitoring the Future, 476.

87. Wilens, T.E., Adler, L.A., Adams, J., Sgambati, S., Rotrosen, J., Sawtelle, R., et al. (2008) Misuse and diversion of stimulants prescribed for ADHD: a systematic

review of the literature. Journal of the American Academy of Child and Adolescent Psychiatry, 47, 21-31.

88. McCabe, S.E. and Boyd, C.J. (2005) Sources of prescription drugs for illicit use. Addictive Behaviors, 30, 1342-50.

89. McCabe, S.E., Knight, J.R., Teter, C.J. and Wechsler, H. (2005) Non-medical use of prescription stimulants among US college students: prevalence and correlates from a national survey. Addiction, 100, 96-106.

90. Babcock, Q. and Byrne, T. (2000) Student perceptions of methylphenidate abuse at a public liberal arts college. Journal of American College Health,, 49, 143-145.

91. Low, K.G. and Gendaszek, A.E. (2002) Illicit use of psychostimulants among college students: A preliminary study. Psychology, Health and Medicine, 7, 283-287.

92. White, B.P., Becker-Blease, K.A. and Grace-Bishop, K. (2006) Stimulant medication use, misuse, and abuse in an undergraduate and graduate student sample. Journal of American College Health, 54, 261-268.

93. Kroutil, L.A., Van Brunt, D.L., Herman-Stahl, M.A., Heller, D.C., Bray, R.M. and Penne, M.A. (2006) Nonmedical use of prescription stimulants in the United States. Drug and Alcohol Dependence, 84, 135-43.

94. Wilens, T.E., Gignac, M., Swezey, A., Monuteaux, M.C. and Biederman, J. (2006) Characteristics of adolescents and young adults with ADHD who divert or misuse their prescribed medications. Journal of American Academy of Child and Adolescent Psychiatry, 45, 408-14.

95. McCabe, S.E., Teter, C.J. and Boyd, C.J. (2004) The Use, Misuse and Diversion of Prescription Stimulants Among Middle and High School Students. Substance Use and Misuse, 39, 1095-1116.

96. Upadhyaya, H.P., Rose, K., Wang, W., O'Rourke, K., Sullivan, B., Deas, D., et al. (2005) Attention-deficit/hyperactivity disorder, medication treatment, and substance use patterns among adolescents and young adults. Journal of Child and Adolescent Psychopharmacology, 15, 799-809.

97. Goldman, L.S., Genel, M., Bezman, R.J. and Slanetz, P.J. (1998) Diagnosis and treatment of attention-deficit/hyperactivity disorder in children and adolescents. Jourbal of the American Medical Association, 279, 1100-1107.

98. Kollins, S.H., MacDonald, E.K. and Rush, C.R. (2001) Assessing the abuse potential of methylphenidate in nonhuman and human subjects: a review. Pharmacology Biochemistry and Behavior, 68, 611-627.

99. Lange, K.W., Reichl, S., Lange, K.M., Tucha, L. and Tucha, O. (2010) The history of attention deficit hyperactivity disorder. Attention Deficit and Hyperactivity Disorders, 2, 241-255.

100. Wenthur, C.J. (2016) Classics in Chemical Neuroscience: Methylphenidate. ACS Chemical Neuroscience, 7, 1030-1040.

101. Cortese, S. (2020) Pharmacologic Treatment of Attention Deficit-Hyperactivity Disorder. New England Journal of Medicine, 383, 1050-1056.

102. Markowitz, J.S. and Patrick, K.S. (2008) Differential pharmacokinetics and pharmacodynamics of methylphenidate enantiomers: does chirality matter? Journal of Clinical Psychopharmacology, 28, S54-61.

103. Faraone, S.V. (2018) The pharmacology of amphetamine and methylphenidate: Relevance to the neurobiology of attention-deficit/hyperactivity disorder and other psychiatric comorbidities. Neuroscience and Biobehavioral Reviews, 87, 255-270.

104. Hodgkins, P., Shaw, M., Coghill, D. and Hechtman, L. (2012) Amfetamine and methylphenidate medications for attention-deficit/ hyperactivity disorder: Complementary treatment options. European Child and Adolescent Psychiatry, 21, 477-92.

105. Markowitz, J.S. and Patrick, K.S. (2008) Differential pharmacokinetics and pharmacodynamics of methylphenidate enantiomers: does chirality matter? Journal of Clinical Psychopharmacology, 28, S54-S61.

106. Williard, R., Middaugh, L., Zhu, H.-J. and Patrick, K. (2007) Methylphenidate and its ethanol transesterification metabolite ethylphenidate: Brain disposition, monoamine transporters and motor activity. Behavioural Pharmacology, 18, 39-51.

107. Markowitz, J.S., DeVane, C.L., Pestreich, L.K., Patrick, K.S. and Muniz, R. (2006) A comprehensive in vitro screening of d-, l-, and dl-threo-methylphenidate: an exploratory study. Journal of Child & Adolescent Psychopharmacology, 16, 687-698.

108. Stevens, T., Sangkuhl, K., Brown, J.T., Altman, R.B. and Klein, T.E. (2019) PharmGKB summary: methylphenidate pathway, pharmacokinetics/pharmacodynamics. Pharmacogenetics and genomics, 29, 136.

109. Kapur, A. (2020) Is Methylphenidate Beneficial and Safe in Pharmacological Cognitive Enhancement? CNS Drugs, 34, 1045-1062.

110. Bartl, J., Palazzesi, F., Parrinello, M., Hommers, L., Riederer, P., Walitza, S., et al. (2017) The impact of methylphenidate and its enantiomers on dopamine synthesis and metabolism in vitro. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 79, 281-288.

111. Heal, D.J. and Pierce, D.M. (2006) Methylphenidate and its isomers: their role in the treatment of attention-deficit hyperactivity disorder using a transdermal delivery system. CNS Drugs, 20, 713-38.

112. Markowitz, J.S., Straughn, A.B. and Patrick, K.S. (2003) Advances in the Pharmacotherapy of Attention-Deficit-Hyperactivity Disorder: Focus on Methylphenidate Formulations. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 23, 1281-1299.

113. Childress, A.C., Komolova, M. and Sallee, F.R. (2019) An update on the pharmacokinetic considerations in the treatment of ADHD with long-acting methylphenidate and amphetamine formulations. Expert Opinion on Drug Metabolism and Toxicology, 15, 937-974.

114. Dinis-Oliveira, R.J. (2017) Metabolomics of Methylphenidate and Ethylphenidate: Implications in Pharmacological and Toxicological Effects. European Journal of Drug Metabolism and Pharmacokinetics, 42, 11-16.

115. Darredeau, C., Barrett Sean, P., Jardin, B. and Pihl Robert, O. (2007) Patterns and predictors of medication compliance, diversion, and misuse in adult prescribed methylphenidate users. Human Psychopharmacology: Clinical and Experimental, 22, 529-536.

116. Ding, Y.S., Fowler, J.S., Volkow, N.D., Dewey, S.L., Wang, G.J., Logan, J., et al. (1997) Chiral drugs: comparison of the pharmacokinetics of [11C]d-threo and l-threo-methylphenidate in the human and baboon brain. Psychopharmacology, 131, 71-78.

117. Riddle, E.L., Hanson, G.R. and Fleckenstein, A.E. (2007) Therapeutic doses of amphetamine and methylphenidate selectively redistribute the vesicular monoamine transporter-2. European journal of Pharmacology, 571, 25-28.

118. Sandoval, V., Riddle, E.L., Hanson, G.R. and Fleckenstein, A.E. (2002) Methylphenidate redistributes vesicular monoamine transporter-2: role of dopamine receptors. Journal of Neuroscience, 22, 8705-8710.

119. Kimko, H.C., Cross, J.T. and Abernethy, D.R. (1999) Pharmacokinetics and clinical effectiveness of methylphenidate. Clinical Pharmacokinetics, 37, 457-70.

120. Wolraich, M.L. and Doffing, M.A. (2004) Pharmacokinetic Considerations in the Treatment of Attention-Deficit Hyperactivity Disorder with Methylphenidate. CNS Drugs, 18, 243-250.

121. Cortese, S., D'Acunto, G., Konofal, E., Masi, G. and Vitiello, B. (2017) New Formulations of Methylphenidate for the Treatment of Attention-Deficit/Hyperactivity Disorder: Pharmacokinetics, Efficacy, and Tolerability. CNS Drugs, 31, 149-160.

122. Patrick, K.S., Straughn, A.B., Minhinnett, R.R., Yeatts, S.D., Herrin, A.E., DeVane, C.L., et al. (2007) Influence of Ethanol and Gender on Methylphenidate Pharmacokinetics and Pharmacodynamics. Clinical Pharmacology and Therapeutics, 81, 346-353.

123. Bentley, J., Snyder, F., Brown, S.D., Brown, R. and Pond, B.B. (2015) Sex differences in the kinetic profiles of d-and l-methylphenidate in the brains of adult rats. European Review for Medical and Pharmacological Sciences, 19, 2514-2519.

124. Food and Drug Administration, 2017b. Cotempla XR-ODT (methylphenidate extended-release orally disintegrating tablets). https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/205489s000lbl.pdf.

125. Zhu, H.-J., Patrick, K.S., Straughn, A.B., Reeves III, O.T., Bernstein, H., Shi, J., et al. (2017) Ethanol interactions with dexmethylphenidate and dl-methylphenidate spheroidal oral drug absorption systems in healthy volunteers. Journal of Clinical Psychopharmacology, 37, 419.

126. Zhu, H.-J., Patrick, K.S. and Markowitz, J.S. (2011) Enantiospecific determination of dl-methylphenidate and dl-ethylphenidate in plasma by liquid chromatography–tandem mass spectrometry: Application to human ethanol interactions. Journal of Chromatography B, 879, 783-788.

127. Thomsen, R., Rasmussen, H.B., Linnet, K. and the, I.C. (2012) Enantioselective Determination of Methylphenidate and Ritalinic Acid in Whole Blood from Forensic Cases Using Automated Solid-Phase Extraction and Liquid Chromatography–Tandem Mass Spectrometry. Journal of Analytical Toxicology, 36, 560-568.

128. Combs, C.C., Hankins, E.L., Copeland, C.L., Brown, S.D. and Pond, B.B. (2013) Quantitative determination of d- and l-threo enantiomers of methylphenidate in brain tissue by liquid chromatography-mass spectrometry. In John Wiley & Sons, Ltd, Great Britain, 1587.

129. Chan, Y., Soldin, S., Swanson, J., Deber, C., Thiessen, J. and Macleod, S. (1980) Gas chromatographic/mass spectrometric analysis of methylphenidate (Ritalin) in serum. Clinical Biochemistry, 13, 266-272.

130. LeVasseur, N.L., Zhu, H.-J., Markowitz, J.S., DeVane, C.L. and Patrick, K.S. (2008) Enantiospecific gas chromatographic–mass spectrometric analysis of urinary methylphenidate: Implications for phenotyping. Journal of Chromatography B, 862, 140-149.

131. Potts, B.D., Martin, C.A. and Vore, M. (1984) Gas-chromatographic quantification of methylphenidate in plasma with use of solid-phase extraction and nitrogen-sensitive detection. Clinical Chemistry, 30, 1374-1377.

132. Lin, S.-N., Andrenyak, D.M., Moody, D.E. and Foltz, R.L. (1999) Enantioselective Gas Chromatography-Negative Ion Chemical Ionization Mass Spectrometry for Methylphenidate in Human Plasma. Journal of Analytical Toxicology, 23, 524-530.

133. Valentine, J.L. and Middleton, R. (2000) GC-MS identification of sympathomimetic amine drugs in urine: rapid methodology applicable for emergency clinical toxicology. Journal of Analytical Toxicology, 24, 211-22.

134. Josefsson, M. and Rydberg, I. (2011) Determination of methylphenidate and ritalinic acid in blood, plasma and oral fluid from adolescents and adults using protein precipitation and liquid chromatography tandem mass spectrometry—A method applied on clinical and forensic investigations. Journal of Pharmaceutical and Biomedical Analysis, 55, 1050-1059.

135. Cantrell, F.L., Ogera, P., Mallett, P. and McIntyre, I.M. (2014) Fatal oral methylphenidate intoxication with postmortem concentrations. Journal of Forensic Science, 59, 847-9.

136. Ramos, L., Bakhtiar, R., Majumdar, T., Hayes, M. and Tse, F. (1999) Liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry enantiomeric separation of dl-threo-methylphenidate, (Ritalin®) using a macrocyclic antibiotic as the chiral selector. Rapid Communications in Mass Spectrometry, 13, 2054-2062.

137. Leis, H.J., Schütz, H. and Windischhofer, W. (2011) Quantitative determination of methylphenidate in plasma by gas chromatography negative ion chemical ionisation mass spectrometry using o-(pentafluorobenzyloxycarbonyl)-benzoyl derivatives. Analytical and Bioanalytical Chemistry, 400, 2663-2670.

138. Seçilir, A., Schrier, L., Bijleveld, Y.A., Toersche, J.H., Jorjani, S., Burggraaf, J., et al. (2013) Determination of methylphenidate in plasma and saliva by liquid chromatography/tandem mass spectrometry. Journal of Chromatography B, 923-924, 22-28.

139. Arvidsson, M., Dahl, M.-L., Beck, O., Ackehed, G., Nordin, K. and Rosenborg, S. (2020) Pharmacokinetics of methylphenidate and ritalinic acid in plasma correlations with exhaled breath and oral fluid in healthy volunteers. European Journal of Clinical Pharmacology, 76, 229-237.

140. Zhu, H.-J., Wang, J.-S., Patrick, K.S., Donovan, J.L., DeVane, C.L. and Markowitz, J.S. (2007) A novel HPLC fluorescence method for the quantification of methylphenidate in human plasma. Journal of Chromatography B, 858, 91-95.

141. Luo, X.-M., Ding, L., Gu, X., Jiang, L.-Y. and Dong, X. (2014) [LC-MS/MS assay of methylphenidate: stability and pharmacokinetics in human]. Yao xue xue bao = Acta pharmaceutica Sinica, 49, 83-88.

142. Zhang, C., Luo, H., Wu, Y., Zhang, J., Zhang, F., Lin, G., et al. (2016) Development and validation of an UFLC-MS/MS method for enantioselectivity determination of d,l-thero-methylphenidate, d,l-thero-ethylphenidate and d,l-thero-ritalinic acid in rat plasma and its application to pharmacokinetic study. Journal of Chromatography B, 1011, 45-52.

143. Bakhtiar, R., Ramos, L. and Tse, F.L.S. (2002) Quantification of methylphenidate in rat, rabbit and dog plasma using a chiral liquid-chromatography/tandem mass spectrometry method: Application to toxicokinetic studies. Analytica Chimica Acta, 469, 261-272.

144. Jiang-hai, L.U., Shan, W., Yang, Q.I.N., Jing, D., You-xuan, X.U. and Mou-tian, W.U. (2009) Confirmation of Methylphenidate and Its Major Metabolite in Human Urine by Liquid Chromatography-Tandem Mass Spectrometry. Jounral of Chinese Mass Spectrometry Society, 267-270.

145. Saito, K., Saito, R. and Ito, R. (2021) Determination of methamphetamine and methylphenidate in urine by liquid chromatography/time-of-flight mass spectrometry coupled with solid-phase dispersive extraction, and its pharmacokinetic application. Forensic Chemistry, 24, 100334.

146. Eichhorst, J., Etter, M., Lepage, J. and Lehotay, D.C. (2004) Urinary screening for methylphenidate (Ritalin) abuse: a comparison of liquid chromatography–tandem

mass spectrometry, gas chromatography–mass spectrometry, and immunoassay methods. Clinical Biochemistry, 37, 175-183.

147. Kwon, W., 서승일, 인문교 and 김진영. (2014) Simultaneous Determination of Methylphenidate, Amphetamine and their Metabolites in Urine using Direct Injection Liquid Chromatography-Tandem Mass Spectrometry. Mass Spectrometry Letters, 5, 104-109.

148. Paterson, S.M., Moore, G.A., Florkowski, C.M. and George, P.M. (2012) Determination of methylphenidate and its metabolite ritalinic acid in urine by liquid chromatography/tandem mass spectrometry. Journal of Chromatography B, 881-882, 20-26.

149. de Cássia Mariotti, K., Rübensam, G., Barreto, F., Bica, V.C., Meneghini, L.Z., Ortiz, R.S., et al. (2014) Simultaneous Determination of Fenproporex, Diethylpropione and Methylphenidate in Oral Fluid by LC-MS/MS. Chromatographia, 77, 83-90.

150. Mulet, C.T., Arroyo-Mora, L.E., Leon, L.A., Gnagy, E. and DeCaprio, A.P. (2018) Rapid quantitative analysis of methylphenidate and ritalinic acid in oral fluid by liquid chromatography triple quadrupole mass spectrometry (LC-QqQ-MS). Journal of Chromatography B, 1092, 313-319.

151. Jang, M., Kim, J., Shin, I., Kang, S., Choi, H. and Yang, W. (2019) Simultaneous determination of methylphenidate and ritalinic acid in hair using LC–MS/MS. Forensic Science International, 294, 183-188.

152. Kintz, P. and Villain, M. (2010) Violence under the influence of methylphenidate as determined by hair analysis. Forensic Toxicology, 28, 115-118.

153. Gandhi, A., Beekman, C., Parker, R., Fang, L., Babiskin, A. and Matta, M.K. (2018) Novel and rapid LC–MS/MS method for quantitative analysis of methylphenidate in dried blood spots. Bioanalysis, 10, 839-850.

154. Markowitz, J.S., Straughn, A.B., Patrick, K.S., DeVane, C.L., Pestreich, L., Lee, J., et al. (2003) Pharmacokinetics of Methylphenidate After Oral Administration of Two Modified-Release Formulations in Healthy Adults. Clinical Pharmacokinetics, 42, 393-401.

155. Beck, O., Stephanson, N., Sandqvist, S. and Franck, J. (2014) Determination of Amphetamine and Methylphenidate in Exhaled Breath of Patients Undergoing Attention-Deficit/Hyperactivity Disorder Treatment. Therapeutic Drug Monitoring, 36.

156. Thomson, M.R., Dowd, J.J., Markowitz, J., Devane, C. and Patrick, K.S. (2002) Enantioselective transesterification of methylphenidate to ethylphenidate after coadministration with ethanol. The Journal of Clinical Pharmacology, 42, 1069-1069.

157. Desrosiers, N.A. and Huestis, M.A. (2019) Oral Fluid Drug Testing: Analytical Approaches, Issues and Interpretation of Results. Journal of Analytical Toxicology, 43, 415-443.

158. Logan, B.K., D'Orazio, A.L., Mohr, A.L.A., Limoges, J.F., Miles, A.K., Scarneo, C.E., et al. (2018) Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities-2017 Update. Journal of Analytical Toxicology, 42, 63-68.

159. Wilson, W. (1995) Supercritical Fluid Chromatography. Journal of Chromatography A, 691, 246.

160. Wang, S.-M., Ling, Y.-C. and Giang, Y.-S. (2003) Forensic applications of supercritical fluid extraction and chromatography. Forensic Science Journal, 2, 5-18.

161. Radcliffe, C., Maguire, K. and Lockwood, B. (2000) Applications of supercritical fluid extraction and chromatography in forensic science. Journal of Biochemical and Biophysical Methods, 43, 261-272.

162. Desfontaine, V., Veuthey, J.-L. and Guillarme, D. (2016) Evaluation of innovative stationary phase ligand chemistries and analytical conditions for the analysis of basic drugs by supercritical fluid chromatography. Journal of Chromatography A, 1438, 244-253.

163. Desfontaine, V., Capetti, F., Nicoli, R., Kuuranne, T., Veuthey, J.-L. and Guillarme, D. (2018) Systematic evaluation of matrix effects in supercritical fluid chromatography versus liquid chromatography coupled to mass spectrometry for biological samples. Journal of Chromatography B, 1079, 51-61.

164. Nováková, L., Grand-Guillaume Perrenoud, A., Francois, I., West, C., Lesellier, E. and Guillarme, D. (2014) Modern analytical supercritical fluid chromatography using columns packed with sub-2μm particles: A tutorial. Analytica Chimica Acta, 824, 18-35.

165. Grand-Guillaume Perrenoud, A., Boccard, J., Veuthey, J.-L. and Guillarme, D. (2012) Analysis of basic compounds by supercritical fluid chromatography:

Attempts to improve peak shape and maintain mass spectrometry compatibility. Journal of Chromatography A, 1262, 205-213.

166. Folprechtová, D., Kalíková, K., Kadkhodaei, K., Reiterer, C., Armstrong, D.W., Tesařová, E., et al. (2021) Enantioseparation performance of superficially porous particle vancomycin-based chiral stationary phases in supercritical fluid chromatography and high performance liquid chromatography; applicability for psychoactive substances. Journal of Chromatography A, 1637, 461846.

167. Midha, K., McKay, G., Rawson, M., Korchinski, E. and Hubbard, J. (2001) Effects of food on the pharmacokinetics of methylphenidate. Pharmaceutical Research, 18, 1185-1189.

168. Markowitz, J.S. and Patrick, K.S. (2013) Ethylphenidate: From biomarker to designer drug. Mental Health Clinician, 3, 318-320.

169. EMCDDA. 2013 Europol 2013 Annual Report on the implementation of Council Decision. http://www.emcdda.europa.eu/attachements.cfm/att_229598_EN_TDAN14001ENN.pdf. (Accessed April 2021)

170. Advisory Committee on the Misuse of Drugs, 2011. Considerations of the novel psychoactive subsances ('Legal Highs'). https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/119139/acmdnps2011.pdf (Accessed April 2021)

171. Gibbons, S. (2012) 'Legal Highs' – novel and emerging psychoactive drugs: a chemical overview for the toxicologist. Clinical Toxicology, 50, 15-24.

172. Bluelight. Ethylphenidate - anyone tried it yet? http://www.bluelight.org/vb/archive/index.php/t-520608.html (Accessed April 2021)

173. EMCDDA. Europol 2011 Annual Report on the implementation of Council Decision. https://www.europol.europa.eu/sites/default/files/publications/emcddaeuropol_annual_report_2011_2012_final.pdf. (Accessed April 2021)

174. Ministry of Health, D., 2013a. Bekendtgørelse om ændring af bekendtgørelse om euforiserende stoffer (Order amending the Order on drugs).

175. Ministry of Health, G., 2013b. Siebenundzwanzigste Verordnung zur Änderung betäubungsmittel-rechtlicher Vorschriften (Twenty-Seventh Regulation amendment of the Narcotics Legislation).

176. Ministry of Health, G., 2013b. Siebenundzwanzigste Verordnung zur Änderung betäubungsmittel-rechtlicher Vorschriften (Twenty-Seventh Regulation amendment of the Narcotics Legislation).

177. 2015. Home Office. The Misuse of Drugs Act 1971 (Temporary Class Drug) Order 2015. http://www.legislation.gov.uk/uksi/2015/1027/pdfs/uksi_20151027_en.pdf. (Accessed April 2021)

178. Ho, J.H., Bailey, G.P., Archer, J.R., Dargan, P.I. and Wood, D.M. (2015) Ethylphenidate: availability, patterns of use, and acute effects of this novel psychoactive substance. European Journal of Clinical Pharmacol, 71, 1185-96.

179. UK Legal Highs Forum. Ethylphenidate/ethylcaine super detailed report. http://www.legalhighsforum.com/showthread.php?17351-Ethylphenidate-Ethylcaine-SUPER-DETAILED-Report (Accessed April 2021)

180. Drugs-forum. 2011. Ethylphenidate experiences. https://drugs-forum.com/threads/ethylphenidate-experiences.156994/ (Accessed April 2021)

181. UK Chemical Research, Thread - Ethylphenidate. https://www.ukchemicalresearch.org/thread-ethylphenidate. (Accessed April 2021).

182. Bluelight. 2013. The Ethylphenidate (Ethyl phenyl(piperidin-2-yl)acetate) Megathread V3. https://www.bluelight.org/xf/threads/the-ethylphenidate-ethyl-phenyl-piperidin-2-yl-acetate-megathread-v3.687516/ (Accessed April 2021)

183. Erwoid, Erwoid expereicen vaults: ethylphenidate reports. https://www.erowid.org/experiences/subs/exp_Ethylphenidate.shtml.

184. Soussan, C. and Kjellgren, A. (2015) “Chasing the High” – Experiences of Ethylphenidate as Described on International Internet Forums. Substance Abuse: Research and Treatment, 9, SART.S22495.

185. Iden, C.R. and Hungund, B.L. (1979) A chemical ionization selected ion monitoring assay for methylphenidate and ritalinic acid. Biomedical Mass Spectrometry, 6, 422-6.

186. Markowitz, J.S., Logan, B.K., Diamond, F. and Patrick, K.S. (1999) Detection of the Novel Metabolite Ethylphenidate After Methylphenidate Overdose With Alcohol Coingestion. Journal of Clinical Psychopharmacology, 19.

187. Portoghese, P.S. and Malspeis, L. (1961) Relative Hydrolytic Rates of Certain Alkyl (b) dl-α-(2-Piperidyl)-phenylacetates. Journal of Pharmaceutical Sciences, 50, 494-501.

188. Patrick, K.S., Corbin, T.R. and Murphy, C.E. (2014) Ethylphenidate as a Selective Dopaminergic Agonist and Methylphenidate–Ethanol Transesterification Biomarker. Journal of Pharmaceutical Sciences, 103, 3834-3842.

189. Parekh, P.K., Ozburn, A.R. and McClung, C.A. (2015) Circadian clock genes: Effects on dopamine, reward and addiction. Alcohol, 49, 341-349.

190. Patrick, K.S., Straughn, A.B., Reeves, O.T., Bernstein, H., Bell, G.H., Anderson, E.R., et al. (2013) Differential Influences of Ethanol on Early Exposure to Racemic Methylphenidate Compared with Dexmethylphenidate in Humans. Drug Metabolism and Disposition, 41, 197-205.

191. Jaffe, S.L. (1991) Intranasal abuse of prescribed methylphenidate by an alcohol and drug abusing adolescent with ADHD. Journal of the American Academy of Child and Adolescent Psychiatry, 30, 773-5.

192. Barrett, S.P. and Pihl, R.O. (2002) Oral methylphenidate-alcohol co-abuse. J Clin Psychopharmacology, 22, 633-4.

193. Wilens, T.E. (2007) The nature of the relationship between attention-deficit/hyperactivity disorder and substance use. Jounal of Clinical Psychiatry, 68 Suppl 11, 4-8.

194. Epstein, T., Patsopoulos, N.A. and Weiser, M. (2014) Immediate-release methylphenidate for attention deficit hyperactivity disorder (ADHD) in adults.

Cochrane Database of Systematic Reviews. doi: 10.1002/14651858.CD005041.pub2.

195. Sun, Z., Murry, D.J., Sanghani, S.P., Davis, W.I., Kedishvili, N.Y., Zou, Q., et al. (2004) Methylphenidate is stereoselectively hydrolyzed by human carboxylesterase CES1A1. Journal of Pharmacology and Experimental Therapeutics, 310, 469-476.

196. Markowitz, J.S., DeVane, C.L., Boulton, D.W., Nahas, Z., Risch, S.C., Diamond, F., et al. (2000) Ethylphenidate Formation in Human Subjects after the Administration of a Single Dose of Methylphenidate and Ethanol. Drug Metabolism and Disposition, 28, 620.

197. Bell, G., Griffin Iii, W. and Patrick, K. (2011) Transdermal and oral dl-methylphenidate – ethanol interactions in C57BL/6J mice: Locomotor activity and brain d-, l-methylphenidate and l-ethylphenidate concentrations. The FASEB Journal, 25, lb427-lb427.

198. Patrick, K.S., Williard, R.L., VanWert, A.L., Dowd, J.J., Oatis, J.E. and Middaugh, L.D. (2005) Synthesis and pharmacology of ethylphenidate enantiomers: the human transesterification metabolite of methylphenidate and ethanol. Journal of Medicinal Chemistry, 48, 2876-2881.

199. Bourland, J.A., Martin, D.K. and Mayersohn, M. (1997) Carboxylesterase-mediated transesterification of meperidine (Demerol) and methylphenidate (Ritalin) in the presence of [2H6]ethanol: preliminary in vitro findings using a rat liver preparation. Journal of Pharmaceutical Sciences, 86, 1494-6.

200. Maskell, P.D., Smith, P.R., Cole, R., Hikin, L. and Morley, S.R. (2016) Seven fatalities associated with ethylphenidate. Forensic Science International, 265, 70-74.

201. Barceló, B., Gomila, I., Rotolo, M., Marchei, E., Kyriakou, C., Pichini, S., et al. (2017) Intoxication caused by new psychostimulants: analytical methods to disclose acute and chronic use of benzofurans and ethylphenidate. International Journal of Legal Medicine, 131, 1543.

202. Nzekoue, F.K., Agostini, M., Verboni, M., Renzoni, C., Alfieri, L., Barocci, S., et al. (2021) A comprehensive UHPLC–MS/MS screening method for the analysis of 98 New Psychoactive Substances and related compounds in human hair. Journal of Pharmaceutical and Biomedical Analysis, 205, 114310.

203. Krueger, J., Sachs, H., Musshoff, F., Dame, T., Schaeper, J., Schwerer, M., et al. (2014) First detection of ethylphenidate in human fatalities after ethylphenidate intake. Forensic Science International, 243, 126-129.

204. Giorgetti, A., Barone, R., Pelletti, G., Garagnani, M., Pascali, J., Haschimi, B., et al. (2021) Development and validation of a rapid LC-MS/MS method for the detection of 182 novel psychoactive substances in whole blood. Drug Testing and Analysis.

205. Adamowicz, P. and Tokarczyk, B. (2019) Screening Analysis for Designer Stimulants by LC-MS/MS. In Langman, L.J. and Snozek, C.L.H. (eds.), LC-MS in Drug Analysis: Methods and Protocols, Springer New York, New York, NY, 165-180.

206. Bailey, G.P., Ho, J.H., Hudson, S., Dines, A., Archer, J.R., Dargan, P.I., et al. (2015) Nopaine no gain: Recreational ethylphenidate toxicity. Clinical Toxicology, 53, 498-499.

207. Parks, C., McKeown, D. and Torrance, H.J. (2015) A review of ethylphenidate in deaths in east and west Scotland. Forensic Science International, 257, 203-208.

CHAPTER II

Chiral Separation and Quantification of *d,l*-Methylphenidate, *d,l*-Ethylphenidate and Ritalinic Acid in Blood by LC-MS/MS[1]

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

[1]Smith CR, Swortwood, MJ. Chiral Separation and Quantification of *d,l*-Methylphenidate, *d,l*-Ethylphenidate and Ritalinic Acid in Blood by LC-MS/MS (2021), as submitted to *Forensic Chemistry*

Abstract

Drugs such as methylphenidate (MPH) are commonly prescribed for Attention-Deficit/Hyperactivity Disorder but may be abused recreationally. *Erythro*-MPH is associated with pharmacological effects and is present as *dextro* (*d*) or *levo* (*l*) configuration, with the *d* configuration being more potent. However, many medications are sold as a racemic mixture and thus analytic separation of isomers is essential. MPH metabolizes into ritalinic acid (RA) as well as ethylphenidate (EPH) in the presence of ethanol. Chiral analysis poses challenges to researchers. Due to limited assays, this project aimed to develop a method that separates and quantifies the enantiomers of MPH and EPH as well as RA in blood. Methods such as this are critical to understanding the pharmacokinetics of chiral cognitive stimulants. This method uses solid-phase extraction and analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and was fully validated. The linear range for MPH and EPH was 0.5-200 ng/mL and 0.5-500 ng/mL for RA. Limit of detection (LOD) was 0.1 ng/mL (with the exception of RA with an LOD of 0.5 ng/mL) and the limit of quantification was 0.5 ng/mL, despite significant matrix effects. Extraction recovery was >79%. Bias was -4.8 to -12.7% and maximum within-run precision was ±12.5% for all analytes. The optimized and validated technique offers chiral separation of the *threo*-enantiomers of *d,l*-MPH, *d,l*-EPH and RA and quantification in blood utilizing LC-MS/MS.

KEY WORDS: Methylphenidate, Cognitive stimulants, Chiral analysis, LC-MS/MS, Solid-phase extraction, Method validation

Introduction

Attention-deficit hyperactivity disorder (ADHD) is a neural brain disease that results from a dopamine deficiency in the synapse of the brain. Patients with ADHD have lower dopamine levels due to increased reuptake transporters. Consequently, decreased dopamine in the brain leads to inattention, boredom, and lack of focus; thus, making it hard for patients to pay attention. Medications combat this disorder by blocking dopamine reuptake transporters in the brain which leads to increased dopamine in the synapse (1-5). One of the main medications to combat this disorder is Ritalin® (6, 7). The active ingredient in Ritalin is methylphenidate (MPH), a cognitive stimulating drug that speeds up brain activity (8). In more recent years, MPH use and abuse have increased making it more relevant when performing toxicology casework (9). Methylphenidate has two chiral centers which gives rise to four stereoisomers: the *d* and *l* configurations of the *threo*- and *erythro*- isomers (10). Studies show that *threo*-MPH is responsible for the pharmaceutical effects of methylphenidate (11). The *d*-enantiomer is predominately responsible for medicinal uses (12-17). However, this medication is typically sold as a racemic mixture of *threo*-MPH with both *d* and *l* enantiomers present (2). As MPH breaks down, the inactive metabolite ritalinic acid (RA) is produced (18, 19) by the CES1 gene in the liver (20). In the presence of ethanol, MPH is converted to ethylphenidate (EPH) (21). Both RA and EPH also contain chiral centers giving rise to multiple stereoisomers and enantiomers. Due to the differing effects of the enantiomers, it is important to study the pharmacodynamics and pharmacokinetics (PD/PK) of this drug (6, 15, 22). For this reason, methods need to be developed, optimized, and validated for chiral separation and quantification of these enantiomers.

There has been extensive research analyzing MPH and its metabolite, RA, using liquid chromatography-mass spectrometry (LC-MS/MS) in blood (23), plasma (23-28), urine (18, 29, 30), oral fluid (8, 23, 25, 27, 31), breath (29), and dried blood spots (32). MPH and RA were isolated from these matrices through a variety of extraction techniques such as liquid-liquid extraction (LLE) (26), protein precipitation (23, 25, 28, 32), or a simple dilution (8, 18, 24, 30). However, these methods were not enantioselective and did not separate the enantiomers of these analytes. Alternatively, there have been methods conducted that have analyzed the enantiomers of MPH using LLE (6, 15) and solid-phase extraction (SPE) (22, 33, 34) for sample preparation. These extraction techniques have been used to isolate *d,l*-MPH from urine (34), brain tissue (33) and traditional matrices such as plasma (6, 15, 34) and blood (22). Of note, Markowitz et al. analyzed ethylphenidate formation in human plasma and urine after consumption of MPH and ethanol. Though separation was non enantioselective, this method quantified MPH, EPH and RA using SPE and LC-MS/MS (34). Thomsen et al. analyzed *d,l*-MPH and RA in blood utilizing SPE following a protein precipitation (22) with a linear range of 0.5 - 500 ng/g. This method was applied to 12 postmortem samples in which *d,l*-MPH and RA were all detected. However, this study did not analyze EPH. There have been few enantioselective methods that have analyzed MPH and both metabolites, RA and EPH. Additionally, LLE is more commonly used to isolate these analytes from various matrices. To the authors knowledge, this is the first known method to extract and quantify RA, as well as the enantiomers of MPH and EPH in blood using SPE and LC-MS/MS in a single assay. For enantioselective separation, crime laboratories typically use derivatization. Due to the harmful nature of derivatizing agents, as well as the expense,

this method aims to separate these enantiomers utilizing a chiral LC column containing vancomycin. The use of vancomycin proves to be cost effective and efficient to achieve baseline separation of *d,l*-MPH and *d,l*-EPH.

Materials and Methods

Chemicals and reagents

Ethanolic solutions of *d,l-threo*-ethylphenidate and ritalinic acid and methanolic solutions of *d,l*-methylphenidate and internal standards (ISTDs), *d,l-threo*-methylphenidate-d10 and *d,l-threo*-ritalinic acid-d10 were purchased from Lipomed (Cambridge, MA, US). Individual enantiomeric standards were not available to assess separately. Defibrinated bovine blood with potassium oxalate and sodium fluoride preservatives was purchased from Quad Five (Ryegate, MT, US) and stored at 4°C. Solid-phase extraction was performed utilizing DAU Clean-Screen (130 mg, 3 mL) SPE columns (United Chemical Technologies, Bristol, PA, US) on an SPEWare System 48™ CEREX Pressure Processor (Baldwin Park, CA, US). A BiotageTurboVap LV Evaporator (Charlotte, NC, US) equipped with nitrogen gas was used for solvent evaporation. Trifluoroacetic acid (99.5%) and ammonium acetate (LC-MS Ultra) were from Fisher Scientific (Hanover Park, IL, US) and acetic acid was from Mallinckrodt Pharmaceuticals (St. Louis, MO, US). Deionized water, ammonium hydroxide and LC-MS grade methanol (>99.9%) used in sample preparation, extraction and mobile phase preparation were purchased from J.T. Baker (Center Valley, MA, US). Dibasic sodium phosphate and monobasic sodium phosphate were from Sigma-Aldrich (St. Louis, MO, US).

Preparation of standard solutions

All analytes were prepared as stock standards in methanol at a concentration of 100,000 ng/mL. ISTD stock standards were prepared in methanol at 10,000 ng/mL. Mixed methanolic solutions were prepared via serial dilution, resulting in concentrations of 5, 10, 50, 100, 500, 1000, 2000, 5000 ng/mL. When fortified in blood, the following concentrations were produced: 0.5, 1, 5, 10, 50, 100, 200, 500 ng/mL. Quality controls (QC) were prepared in solution at three concentrations. For MPH, EPH and RA, the low and medium QCs were 15 and 250 ng/mL, respectively. The high QC was 1500 and 4000 ng/mL for MPH/EPH and RA, respectively. When fortified in blood, the low and medium QCs for MPH, EPH and RA were 1.5 and 25 ng/mL, respectively. The high QC was 150 and 400 ng/mL for MPH/EPH and RA, respectively. A mixed methanolic internal standard solution was prepared at 100 ng/mL (10 ng/mL in blood). All solutions were stored at -20°C in amber vials.

Solid-phase extraction

Blood (250 µL) was fortified with 25 µL of calibrator or QC solution. All samples and negative controls were fortified with 25 µL of ISTD. Phosphate buffer (100mM, pH 6, 1mL) was added to all samples and vortexed. The mixtures were centrifuged (2000rpm, 10 min) before loading onto the SPE column pre-conditioned with methanol (1 mL) and phosphate buffer (1 mL). After sample loading, the SPE columns were washed with acetic acid (0.1 M, 1 mL) and methanol (1 mL). The columns were dried under nitrogen at maximum pressure for 10 min. Analytes were eluted with 2% ammonium hydroxide in methanol (2 mL). The solutions were evaporated to dryness under nitrogen at 50°C. Samples were reconstituted in 100 µL mobile phase (2:98) before

being transferred to LC autosampler vials. A total of 1 µL was injected onto the LC-MS/MS.

Instrumentation

Liquid chromatography

Analysis was performed on an Agilent 1290 Infinity II Liquid Chromatograph coupled to an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA). Separation of analytes was achieved using an Agilent Poroshell Chiral-V column (2.7 µm, 2.1 x 100 mm) with a Poroshell 120 EC-C18 (2.7 µm, 2.1 x 5 mm) guard held at 35°C. An isocratic elution was used with mobile phase A:B at 2:98 at a 0.6 mL/min flow rate. Aqueous mobile phase (A) was deionized water. Organic mobile phase (B) consisted of 0.0125% trifluoroacetic acid (v/v) and 0.025% ammonium acetate (w/v) in methanol. Total run time was 4 min.

Mass spectrometry

Positive mode electrospray ionization was used. Analytes were detected with multiple reaction monitoring (MRM) with one transition for quantification and one transition for qualification per compound. The gas temperature and gas flow were set at 300°C and 5 L/min, respectively. The nebulizer was at 45 psi. The sheath gas was at 350°C with a flow of 11 L/min. The capillary voltage was at 3500 V. The optimized MS/MS parameters are summarized in Table 2.1.

Table 2.1. Optimized acquisition parameters for analyte quantification

Analyte	Retention Time (min)	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion *(m/z)*	Paired Internal Standard
RA	0.66	220.1	84.0	56.1	RA-d10
l-MPH	1.09	234.1	84.1	56.0	*l*-MPH-d10
d-MPH	1.36	234.1	84.1	56.0	*d*-MPH-d10
l-EPH	0.98	248.2	84.1	56.0	*l*-MPH-d10
d-EPH	1.12	248.2	84.1	56.0	*d*-MPH-d10
d-MPH-d10	1.40	244.2	93.1	61.1	-
l-MPH-d10	1.12	244.2	93.1	61.1	-
RA-d10	0.65	230.1	93.1	61.1	-

Method validation

Method validation was performed according to the Standard Practices for Method Validation in Forensic Toxicology published by the AAFS Standards Board (ASB) as a guideline (ANSI/ASB Standard 036) (35). Calibration models were determined using 8 non-zero calibrators over 5 days using different sources of blood. Linearity was assessed using the least squares model and considered acceptable when $R^2 > 0.99$. Limits of detection (LOD) and limits of quantification (LOQ) were analyzed in triplicate over three days in three different sources of blood. LOD were evaluated in terms of signal to noise ratio of ≥3 and ion ratios (within ±20%). LOQ were considered acceptable with signal to noise ratio of ≥10 and bias and precision within ±20%.

Bias and precision were evaluated at three QC concentrations in triplicate over 5 days. Bias was considered acceptable within ±20%. Within-run and between run precision (% coefficient of variation, CV) were calculated at each QC concentration and considered acceptable within ±20%. Carryover was assessed on three days by injecting a blank matrix sample immediately after injection of the highest calibrator. Carryover was considered negligible if peaks were below the method's LOD.

Endogenous interferences were determined by injection of extracted blank matrix with ISTD from 5 sources. Negative control samples were fortified with ISTD only and examined for the presence of d0 analytes. Exogenous interferences (10,000 ng/mL) were fortified into low QC samples (n=3) and extracted following the described procedure. The compounds evaluated included Δ^9-tetrahydrocannabinol, alprazolam, amobarbital, amphetamine, amitriptyline, butalbital, caffeine, carbamazepine, carisoprodol, cocaine, codeine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, diphenhydramine,

hydrocodone, hydromorphone, ketamine, methadone, nicotine, nordiazepam, oxazepam, oxycodone, pentobarbital, phencyclidine, phenobarbital, propoxyphene, secobarbital, tetrahydrocannabinolic acid, tramadol, and zolpidem. Interferences were considered negligible if there were no interfering peaks and if the targeted QC analytes (MPH, RA, EPH) quantified within ±20%.

Matrix effects (ion suppression/enhancement) were determined using post-extraction addition using low and high QCs in 10 sources of matrix. Matrix effects were calculated by dividing the mean response of the post-extraction fortified samples by the mean response of the neat standards. Extraction recovery was determined using post-extraction addition in 5 sources of matrix at 10 ng/mL. Recovery was calculated by dividing the mean response of the post-extraction fortified samples by the mean response of the pre-extraction fortified samples. Matrix effects were considered acceptable within ±25%

A 1:10 dilution of high QC was performed in triplicate. Short-term stability was assessed in triplicate at low and high QC concentrations under the following: processed autosampler (4°C, 48 hours), refrigerated (4°C, 48 hours) and room temperature (20°C, 24 hours). Dilution and stability were considered acceptable if bias was within ±20%.

Results and Discussions

Method development

Extensive method development sought to optimize an extraction with minimal interferences and maximal instrument response for the analytes of interest. Due to the focus of this study being enantiomeric separation, various LC columns were used to achieve this. Ultimately, Agilent Poroshell Chiral-V allowed for separation all three

analytes as well as full enantiomeric separation of *d,l*-MPH, *d,l*-EPH and *d,l*-MPH-d10. The vancomycin protein within the column contains 18 chiral centers, 5 aromatic rings and 3 cavities in which the enantiomers can get trapped (by stereoselectivity) leading to separation (36). This protein is suggested for use when performing reverse-phase LC separation of amines. Chromatographic separation was initially investigated using mobile phase A as 0.1% formic acid in water and mobile phase B as 0.01% in methanol. During this optimization, alternative additives, including ammonium formate, trifluoroacetic acid and ammonium acetate, were evaluated in attempt to separate the enantiomers. In addition, acetonitrile was also investigated as the organic solvent mobile phase. Of all mobile phase compositions analyzed, high organic mobile phase compositions allowed for separation of the enantiomers of *d,l*-MPH and *d,l*-EPH. Ultimately, trifluoro acetic acid (0.0125%, v/v) and ammonium acetate (0.025%, w/v) in methanol was selected as described by Zhu et. al (6). Mobile phase A consisted of unmodified deionized water. The final composition used was mobile phase A:B at 2:98 using an isocratic elution. This allowed for near baseline resolution of the MPH and EPH.

A method to simultaneously extract both the alkaline and acidic analytes was investigated using various SPE columns, aqueous and organic washes, and elution solvents. Ultimately, the UCT DAU-Clean Screen columns successfully extracted the target analytes from blood. Various elution solutions were analyzed, including dichloromethane with isopropyl alcohol, methanol and ethyl acetate with multiple percentages of ammonium hydroxide. Ultimately, 2% ammonium hydroxide in methanol was chosen as it resulted in the highest analytical recoveries. Lastly, the reconstitution volume and injection volume were optimized. Samples (extracted as described above)

were reconstituted in mobile phase at 100, 500, or 1000µL and injected at a range of 1-10µL. Peak shape and area were considered when determining optimal conditions. A small reconstitution volume (100µL) with a small injection volume (1µL) resulted in the best peak shape and response while minimizing matrix effects and allowing for reinjection, if necessary.

Method validation

Least squares regression was used with 8 (RA) and 7 non-zero calibrators (MPH and EPH). All calibration curves resulted in R2 values of ≥0.997 using a 1/x weighting. Analyte response was linear from the LOQ to 500 ng/mL for RA and from the LOQ to 200 ng/mL for MPH and EPH. The method currently presented displays a linear range that is inclusive to concentrations found in literature. Thomsen et al. reported a sum concentration of 5-89 ng/g of methylphenidate (22) while Josefsson et al. and Schulz et. al reported a range of 10-60 ng/mL and trace-95 ng/mL (23, 37). These ranges have all been reported to be within therapeutic concentrations in living subjects. Calibration data from five days are summarized in Table 2.2 for RA, MPH, and EPH.

Table 2.2. Summary of limit of detection (LOD), limit of quantitation (LOQ), linear range, and mean values of R^2, slope and y-intercept for all five analytes in blood

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Calibration Range (ng/mL)	R^2 (n=5)	Slope (n=5)	Y-intercept (n=5)
RA	0.5	0.5	0.5 - 500	0.9987	1.4251	0.1757
l-MPH	0.1	0.5	0.5 - 200	0.9991	1.6902	0.5403
d-MPH	0.1	0.5	0.5 - 200	0.9979	1.7523	0.5773
l-EPH	0.1	0.5	0.5 - 200	0.9991	1.5672	0.4154
d-EPH	0.1	0.5	0.5 - 200	0.9979	1.9742	0.6102

The LOD and LOQ were 0.1 and 0.5 ng/mL for MPH and EPH, respectively. The LOD and LOQ were 0.5 ng/mL for RA. The LOD and LOQ were determined to be

acceptable for this study as they encompass the vales found in literature. These LOD/LOQ values are comparable to the study done by Thomsen et al. who obtained a LOQ of 0.5 ng/g (22). At the LOQ over three days, bias ranged from -3.1 to -17.2 % and precision were 1.8 to 16.9 %CV. Bias and precision results for three QC concentrations are summarized in Table 2.3 and were considered acceptable. For all analytes, bias was <±12.7%. Between-run precision was 3.6 to 6.9 %CV. Maximum within-run precision was 4.7 to 12.5 % CV.

Table 2.3. Summary of bias, between-run precision, and maximum within-run precision in blood at three quality control (QC) concentrations over the linear range

Analyte	Bias (%, n=15)			Between Run Precision (%CV, n=15)			Maximum Within Run Precision (%CV, n=5)		
	LQC[1]	MQC[2]	HQC[3]	LQC	MQC	HQC	LQC	MQC	HQC
l-MPH	-10.1	-7.9	-8.9	5.5	4.1	4.9	7.5	4.9	7.6
d-MPH	-10.7	-6.4	-8.6	6.0	5.6	5.2	8.9	5.2	8.9
l-EPH	-12.7	-10.3	-10.3	5.0	3.6	4.8	8.7	4.7	7.3
d-EPH	-11.5	-7.1	-10.2	6.9	6.9	5.9	12.5	5.6	9.4
RA	-9.5	-4.8	-7.1	5.6	5.0	6.1	5.4	9.1	4.7

[1]Low QC concentration: 1.5 ng/mL
[2]Medium QC concentration: 25 ng/mL
[3]High QC concentration: 150 ng/mL (MPH and EPH), 400 ng/mL (RA)

Matrix effects ranged from -48.2 to 48.7% at the low concentration and -55.6 to 24.2% at the high concentration. RA displayed ion suppression while MPH and EPH exhibited enhancement. Recovery ranged from 78.6 to 89.8% for all analytes at a concentration of 10 ng/mL. When assessing matrix effects, though the values fall out of the recommended ±25% range as suggested in the validation standard, the deuterated ISTD compensated for this phenomenon and was considered appropriately matched. Additionally, matrix effects were considered acceptable as the %CV values demonstrated reproducibility. Similarly, Thomsen et al. also observed matrix effects in blood for some

analytes while Zhu et al. experienced no matrix effects in plasma (6, 22). Whole blood consists of proteins, cells and other endogenous components that contribute to enhanced matrix effects. Therefore, these values were considered acceptable for this method. These data are summarized in Table 2.4.

Table 2.4. Matrix effects (%) at two quality control (QC) concentrations (n=10) and recovery at a concentration of 10 ng/mL in blood for all five analytes

Analyte	Matrix Effects (%CV)		Recovery (%, n=5)
	LQC[1]	HQC[2]	
RA	-48.3 (14.3)	-55.6 (10.6)	89.8
l-MPH	44.5 (6.4)	20.4 (10.3)	84.0
d-MPH	24.0 (6.3)	4.8 (11.2)	89.0
l-EPH	44.6 (6.4)	21.6 (10.3)	88.1
d-EPH	48.7 (6.7)	24.2 (9.9)	78.6
RA-d10	-47.2 (13.4)	-54.7 (10.8)	89.5
l-MPH-d10	44.7 (6.3)	19.5 (9.1)	88.3
d-MPH-d10	28.6 (6.4)	4.8 (11.7)	80.2

[1]Low QC concentration: 1.5 ng/mL
[2]High QC concentration: 150 ng/mL (MPH and EPH), 400 ng/mL (RA)

Endogenous interferences were evaluated in blank blood. No analyte peaks or interfering peaks were detected in the blank samples. Negative samples, with ISTD only, were evaluated for presence of d0 analytes and no peaks were detected. Common drugs of abuse were evaluated for interferences by evaluating 32 basic, acidic and neutral drugs. The LQC samples successfully quantified within ±20% (range: -20.0 to -11.5%) demonstrating selectivity of the method. No carryover was observed as analytes were evaluated to determine if they met or exceeded LOD criteria. Dilution integrity bias was -19.11 to 5.5% for all analytes and was acceptable.

For processed sample stability in the autosampler, bias ranged from -16.8 to -6.0% and were considered acceptable, as described in Table 2.5. For room temperature stability, RA was considered stable at the high concentration for 24 hours while the enantiomers of MPH and *l*-EPH demonstrated instability at both concentrations at 24 hours. At the high concentration, *d*-EPH was considered stable for 24 hours but was considered unstable at the low concentration. Overall, bias ranged from -54.7 to 35.5% for all analytes. For refrigerated stability, RA remained stable for 48 hours while *d,l*-MPH and *l*-EPH were unstable after 48 hours Like room temperature, *d*-EPH remained stable for 48 hours at the high concentration but unstable at the low concentration. Overall bias ranged from -27.8 to -2.5% for all analytes. When analyzing stability of these compounds, a general trend can be observed when comparing to a long-term study conducted by Smith *et al.* The same analytes in blood were stored under refrigerated and room temperature conditions over a nine-month period at a low (15 ng/mL) and high (150 ng/mL) concentration (38). The results are comparable as they reported short-term stability (~1 week) when blood was stored under refrigeration. They also noted MPH and EPH degradation at room temperature and increasing RA concentrations. Their study confirmed MPH breaks down to RA when blood was not stored under frozen conditions. The rate of degradation varies slightly between the two studies and may be due to age of blood source and difference in concentrations. Given these data, storage conditions play an important role in preserving analyte concentration in blood.

Table 2.5. Fortified and processed sample stability at the low QC and high QC in blood stored under various conditions. Bold numbers indicate values outside of acceptable range (±20%)

	Stability (%Bias, n=3)					
Analyte	Autosampler (Processed, 24h, 4°C)		Room Temperature (24h, 24°C)		Refrigerator (48h, 4°C)	
	LQC[1]	HQC[2]	LQC	HQC	LQC	HQC
RA	-6.0	-6.3	**35.5**	15.2	-11.9	-2.5
l-MPH	-10.6	-6.5	**-54.7**	**-44.3**	**-22.6**	**-24.8**
d-MPH	-8.3	-6.7	**-54.0**	**-40.2**	**-27.8**	**-23.8**
l-EPH	-16.6	-9.8	**-37.4**	**-30.6**	**-23.4**	**-21.2**
d-EPH	-16.8	-10.3	**-28.6**	-19.0	**-21.9**	-18.7

[1]Low QC concentration: 1.5 ng/mL
[2]High QC concentration: 150 ng/mL (MPH and EPH), 400 ng/mL (RA)

Conclusion

The present method was developed and optimized for the chiral separation and detection of *d,l*-MPH and its metabolites, *d,l*-EPH and RA. The method was validated following ASB/ANSI Standard 036. Calibration models, LOD, LOQ, bias and precision, interferences and carryover were considered acceptable. Despite ion enhancement and suppression, internal standards compensated appropriately, and matrix effects were reproducible and did not negatively impact LOQ. Dilution integrity was sustained for all analytes a factor of 1:10. Lastly, all analytes were stable in the autosampler after processing.

The major goal of this study was to separate the enantiomers of *d,l*-MPH and *d,l*-EPH and quantify them individually. With this, future studies assess the pharmacokinetics and pharmacodynamics of these enantiomers in the body. Additionally, metabolism studies can be conducted to better understand the breakdown of each parent drug enantiomer to its metabolites. This is the first method (to our knowledge) that

separates and quantifies the enantiomers of *d,l*-MPH and *d,l*-EPH with RA in the same assay in blood using LC-MS/MS.

References

1. Ritz, M.C., Lamb, R.J., Goldberg, S.R. and Kuhar, M.J. (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science, 237, 1219-1223.
2. Ramos, L., Bakhtiar, R. and Tse, F.L.S. (2000) Liquid-liquid extraction using 96-well plate format in conjunction with liquid chromatography/tandem mass spectrometry for quantitative determination of methylphenidate (Ritalin®) in human plasma. Rapid Communications in Mass Spectrometry, 14, 740-745.
3. Patrick, K.S. and Markowitz, J.S. (1997) Pharmacology of methylphenidate, amphetamine enantiomers and pemoline in attention-deficit hyperactivity disorder. Human Psychopharmacology-Clinical and Experimental, 12, 527-546.
4. Wenthur, C.J. (2016) Classics in Chemical Neuroscience: Methylphenidate. ACS Chemical Neuroscience, 7, 1030-1040.
5. Wilens, T.E., Morrison, N.R. and Prince, J. (2011) An update on the pharmacotherapy of attention-deficit/hyperactivity disorder in adults. Expert Review of Neurotherapeutics, 11, 1443-65.
6. Zhu, H.-J., Patrick, K.S. and Markowitz, J.S. (2011) Enantiospecific determination of dl-methylphenidate and dl-ethylphenidate in plasma by liquid chromatography–tandem mass spectrometry: Application to human ethanol interactions. Journal of Chromatography B, 879, 783-788.
7. Arnold, L.E. (2000) Methyiphenidate vs. amphetamine: Comparative review. Journal of Attention Disorders, 3, 200-211.

8. Mulet, C.T., Arroyo-Mora, L.E., Leon, L.A., Gnagy, E. and DeCaprio, A.P. (2018) Rapid quantitative analysis of methylphenidate and ritalinic acid in oral fluid by liquid chromatography triple quadrupole mass spectrometry (LC-QqQ-MS). Journal of Chromatography B, 1092, 313-319.

9. Wu, L.-T., Pilowsky, D.J., Schlenger, W.E. and Galvin, D.M. (2007) Misuse of methamphetamine and prescription stimulants among youths and young adults in the community. Drug and Alcohol Dependence, 89, 195-205.

10. Markowitz, J.S., Straughn, A.B. and Patrick, K.S. (2003) Advances in the Pharmacotherapy of Attention-Deficit-Hyperactivity Disorder: Focus on Methylphenidate Formulations. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 23, 1281-1299.

11. Szporny, L. and Görög, P. (1961) Investigations into the correlations between monoamine oxidase inhibition and other effects due to methylphenydate and its stereoisomers. Biochemistry, 8, 263-268.

12. Ding, Y.S., Fowler, J.S., Volkow, N.D., Dewey, S.L., Wang, G.J., Logan, J., et al. (1997) Chiral drugs: comparison of the pharmacokinetics of [11C]d-threo and l-threo-methylphenidate in the human and baboon brain. Psychopharmacology, 131, 71-78.

13. Ding, Y.-S., Gatley, S.J., Thanos, P.K., Shea, C., Garza, V., Xu, Y., et al. (2004) Brain kinetics of methylphenidate (Ritalin) enantiomers after oral administration. Synapse, 53, 168-175.

14. Patrick, K.S., Caldwell, R.W., Ferris, R.M. and Breese, G.R. (1987) Pharmacology of the enantiomers of threo-methylphenidate. Journal of Pharmacology and Experimental Therapeutics, 241, 152-158.

15. Ramos, L., Bakhtiar, R., Majumdar, T., Hayes, M. and Tse, F. (1999) Liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry enantiomeric separation of dl-threo-methylphenidate, (Ritalin®) using a macrocyclic antibiotic as the chiral selector. Rapid Communcations, 13, 2054-2062.

16. Markowitz, J.S. and Patrick, K.S. (2008) Differential pharmacokinetics and pharmacodynamics of methylphenidate enantiomers: does chirality matter? Journal of Clinical Psychopharmacology, 28, S54-61.

17. Bakhtiar, R., Ramos, L. and Tse Francis, L.S. (2003) Toxicokinetic assessment of methylphenidate (Ritalin®) in a 13-week oral toxicity study in dogs. Biomedical Chromatography, 18,45-50.

18. Paterson, S.M., Moore, G.A., Florkowski, C.M. and George, P.M. (2012) Determination of methylphenidate and its metabolite ritalinic acid in urine by liquid chromatography/tandem mass spectrometry. Journal of Chromatography B, 881-882, 20-26.

19. Srinivas, N.R., Hubbard, J.W., Korchinski, E.D. and Midha, K.K. (1992) Stereoselective Urinary Pharmacokinetics of dl-threo-Methylphenidate and Its Major Metabolite in Humans. Journal of Pharmaceutical Sciences, 81, 747-749.

20. Srinivas, N.R., Hubbard, J.W., McKay, G., Hawes, E.M. and Midha, K.K. (1991) In vitro hydrolysis of RR,SS-threo-methylphenidate by blood esterases--differential and enantioselective interspecies variability. Chirality, 3, 99-103.

21. Patrick, K.S., Straughn, A.B., Minhinnett, R.R., Yeatts, S.D., Herrin, A.E., DeVane, C.L., et al. (2007) Influence of Ethanol and Gender on Methylphenidate Pharmacokinetics and Pharmacodynamics. International Journal of Clinical Pharmacology Therapeutics, 81, 346-353.

22. Thomsen, R., Rasmussen, H.B., Linnet, K. and the, I.C. (2012) Enantioselective Determination of Methylphenidate and Ritalinic Acid in Whole Blood from Forensic Cases Using Automated Solid-Phase Extraction and Liquid Chromatography–Tandem Mass Spectrometry. Journal of Analytical Toxicology, 36, 560-568.

23. Josefsson, M. and Rydberg, I. (2011) Determination of methylphenidate and ritalinic acid in blood, plasma and oral fluid from adolescents and adults using protein precipitation and liquid chromatography tandem mass spectrometry—A method applied on clinical and forensic investigations. Journal of Pharmaceutical and Biomedical Analysis, 55, 1050-1059.

24. Luo, X.-M., Ding, L., Gu, X., Jiang, L.-Y. and Dong, X. (2014) [LC-MS/MS assay of methylphenidate: stability and pharmacokinetics in human]. Yao xue xue bao = Acta Pharm Sin 49:83-88.

25. Seçilir, A., Schrier, L., Bijleveld, Y.A., Toersche, J.H., Jorjani, S., Burggraaf, J., et al. (2013) Determination of methylphenidate in plasma and saliva by liquid

chromatography/tandem mass spectrometry. Journal of Chromatography B, 923-924, 22-28.

26. Kasabova, L. and Svinarov, D. (2015) Determination of Methylphenidate In Human Plasma By A Validated Lc-Ms/Ms Method. Clinical Therapeutics, 37, e62-e63.

27. Preiskorn, J., Studer, S., Rauh, R., Lukačin, R., Geffert, C., Fleischhaker, C., et al. (2018) Inter- and Intraindividual Variation of Methylphenidate Concentrations in Serum and Saliva of Patients with Attention-Deficit/Hyperactivity Disorder. Therapeutic Drug Monitoring, 40, 435-442

28. Studer, S., Burghardt, S., Fleischhaker, C., Schulz, E., Clement, H.W. and Lukačin, R. (2014) Methylphenidate and ritalinic acid determination in serum and saliva from patients with attention deficit hyperactivity disorder. Pharmacopsychiatry, 47, A4.

29. Beck, O., Stephanson, N., Sandqvist, S. and Franck, J. (2014) Determination of Amphetamine and Methylphenidate in Exhaled Breath of Patients Undergoing Attention-Deficit/Hyperactivity Disorder Treatment. Therapeutic Drug Monitoring, 36.

30. Eichhorst, J., Etter, M., Lepage, J. and Lehotay, D.C. (2004) Urinary screening for methylphenidate (Ritalin) abuse: a comparison of liquid chromatography–tandem mass spectrometry, gas chromatography–mass spectrometry, and immunoassay methods. Clinical Biochemistry, 37, 175-183.

31. Studer, S., Preiskorn, J., Lukacin, R., Geffert, C., Fleischhaker, C., Clement, H.W., et al. (2016) Inter- and intraindividual variations of methylphenidate in serum and oral fluid of ADHS patients. Pharmacopsychiatry, 26, P12.

32. Gandhi, A., Beekman, C., Parker, R., Fang, L., Babiskin, A. and Matta, M.K. (2018) Novel and rapid LC–MS/MS method for quantitative analysis of methylphenidate in dried blood spots. Bioanalysis. 10, 839-850.

33. Combs Carolyn, C., Hankins Erin, L., Copeland Cara, L., Brown Stacy, D. and Pond Brooks, B. (2013) Quantitative determination of d- and l-threo enantiomers of methylphenidate in brain tissue by liquid chromatography–mass spectrometry. Biomedical Journal, 27,1587-1589.

34. Markowitz, J.S., DeVane, C.L., Boulton, D.W., Nahas, Z., Risch, S.C., Diamond, F., et al. (2000) Ethylphenidate Formation in Human Subjects after the Administration of a Single Dose of Methylphenidate and Ethanol. Drug Metabolism, 28, 620.

35. ASB/ANSI. (2019) ANSI/ASB Standard 036, First Edition. In Standard Practices for Method Validation in Forensic Toxicology.

36. Alothman, Z.A., Alanazi, A.G., Suhail, M. and Ali, I. (2020) HPLC enantio-separation and chiral recognition mechanism of quinolones on vancomycin CSP. Journal of Chromatography B, 1157, 122335.

37. Schulz, M., Iwersen-Bergmann, S., Andresen, H. and Schmoldt, A. (2012) Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. Critical Care, 16, R136.

38. Smith, C.R. and Swortwood, M.J. (2021) Short- and Long-Term Stability of Methylphenidate and Its Metabolites in Blood. Journal of Analytical Toxicology, 45, 863-869.

CHAPTER III

Analysis of Methylphenidate and Other Cognitive Stimulants in Oral Fluid by LC-MS/MS[2]

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

[2]Smith CR, Swortwood, MJ. Analysis of methylphenidate and other cognitive stimulants in oral fluid by LC-MS/MS (2021), as submitted to *Journal of Forensic Sciences*

Abstract

Oral fluid is an alternative matrix that has proven to be useful for detection of drugs. Oral fluid is easy to collect, non-invasive, and may indicate recent drug use. There are limited methods available that analyze cognitive stimulants in oral fluid. Cognitive stimulants are used to treat attention-deficit/hyperactivity disorder (ADHD), a neurological disorder that emerges from lack of dopamine in the brain. To combat this disorder, medications inhibit dopamine reuptake by blocking transporters in the brain. Though commonly diagnosed in children, ADHD may extend beyond adolescence and abuse of medications in college students is not uncommon. The goal of this study was to develop and validate a quantitative method for methylphenidate, ethylphenidate, lisdexamfetamine and amphetamine in oral fluid using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The linear range was 0.5-100 ng/mL (with the exception of lisdexamfetamine at 5-500 ng/mL). Bias and between-run precision were considered acceptable at ±11.0% bias and ±12.2%CV. No interferences or carryover were observed and dilution integrity was sustained at a factor of 1:10. This validated method was applied to four authentic oral fluid samples collected with a Quantisal® device from college students. Lisdexamfetamine and amphetamine were quantified at 5.8 ng/mL and 6.0-78.8 ng/mL, respectively. This is the first known method to quantify these analytes in oral fluid using LC-MS/MS and may give rise to interpretive value in a forensic toxicology setting.

KEY WORDS: Cognitive stimulants, LC-MS/MS, Oral fluid

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is neurological disorder that arises from dopamine deficiency in the synapse. In the brain, dopamine levels are decreased due to an increase of reuptake transporters which leads to inattention, boredom, and lack of focus. This inability to pay attention leads to ADHD diagnosis. Medications work to combat this disorder by blocking dopamine reuptake transporters in the synapse which leads to an increase in dopamine, speeding up brain activity (1-6). There are many medications that are prescribed to treat ADHD. Common medications include Ritalin®, Adderall®, Vyvanse®, and Concerta® among others. The active ingredients in these medications are methylphenidate (MPH) (Ritalin® and Concerta®), amphetamine (AMP) (Adderall®, Adzenys®, and Mydayis®), and lisdexamfetamine (LDX) (Vyvanse®) (7-9). Depending on the severity of the disorder, prescriptions can be short-, intermediate- or long-acting stimulants and can last 3-12 hours. There are also long-acting non-stimulants such as Strattera® or Intuniv® that can last 12-24 hours (10, 11). Cognitive stimulants are traditionally prescribed in children but ADHD has been classified as the fastest growing disability on the college campus (12). From 2003-2017, the National Survey on Drug Use from Monitoring the Future showed that misuse of methylphenidate in college aged students rose from 1.4% to 5.7% (13). These medications have a high potential for abuse at the collegiate level as the stimulant effect can increase attention and combat boredom in the classroom and speed up brain activity for tests. Due to this recent trend, it is important for forensic scientists to detect these cognitive stimulants in biological matrices.

There is limited research analyzing cognitive stimulants in oral fluid utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS). Though blood and urine are traditional matrices in forensic toxicology, oral fluid has emerged as a viable alternative matrix. Oral fluid collection is quick, non-invasive and has limited biohazard risks (14, 15). Methylphenidate has been detected in oral fluid after ingestion of MPH medications in clinical settings. Josefsson et al. collected oral fluid from 5 adult and 5 adolescent patients with known ADHD that were prescribed 36-72 mg/day MPH dose (16). OF was collected 6 hours after intake and mean MPH concentrations were 44 ng/mL in adolescents and 34 ng/mL in adults (16). Similarly, Marchei et al. dispensed a 20 mg tablet of MPH and maximum concentrations (C_{max}) in oral fluid were 16.2-87.3 ng/mL (17). In a clinical application, Mulet et al. collected 149 oral fluid samples from children diagnosed with ADHD and MPH concentrations were 3.5-61.3 ng/mL (18). Among the few methods that have been validated in oral fluid using LC-MS/MS, the limit of detection (LOD) and the limit of quantification (LOQ) has ranged from 0.1-0.5 ng/mL and 0.1-2.5 ng/mL, respectively (16-20). Like MPH, lisdexamfetamine was detected, with its metabolite *d*-amphetamine (AMP), after ingestion of a cognitive stimulant medication. Comiran et al. validated a method for LDX and AMP detection with a LOQ of 1 ng/mL. A single 70 mg tablet of LDX was administered and oral fluid concentrations of LDX and AMP were 16.3 ng/mL and 131.6 ng/mL, respectively, two hours post-administration (21). Following the same study design in a follow-up pharmacodynamic and pharmacokinetic study of LDX and *d*-AMP, subjects were given a single 70 mg tablet of MPH (22). OF was collected at 16 time points over 72 hours and the C_{max} of LDX and AMP were 15.4 ng/mL and 363 ng/mL, respectively (22). Similarly,

Bottcher et al. validated a method for LDX and applied this method (LOD: 0.0059 ng/mL and LOQ: 0.0072 ng/mL) to 102 samples (average dose of 68 mg). Concentrations of LDX and AMP were 0.01-2895 ng/mL and 2-8410 ng/mL, respectively (23). Due to the limited assays, comprehensive methods need to be developed and validated for detection and quantification of these analytes in oral fluid.

Additionally, due to the ease of collection, oral fluid testing includes the potential of on-site testing which may be advantageous for driving under the influence of drugs (DUID) cases. Zanacaro et al. collected oral fluid samples from drivers and MPH was detected from 3-18.2 ng/mL (20). Non-traditional cognitive stimulants related to ADHD were also detected in drivers. Ethylphenidate (EPH), typically considered a metabolite of MPH in the presence of alcohol, has also been detected as a novel psychoactive substance (NPS) in French drivers from 2016-2020 (24). Due to limited methods detecting these cognitive stimulants in oral fluid, the aim of this study was to develop and validate a comprehensive method for MPH, EPH, LDX and AMP in oral fluid using LC-MS/MS. Additionally, this method was applied to four authentic oral fluid samples from college students to demonstrate forensic applicability. This is the first method to the author's knowledge that analyzes all four analytes in a single assay using oral fluid.

Materials and Methods

Chemicals and reagents

Certified reference standards *d,l*-methylphenidate and *d,l*-ethylphenidate, and deuterated internal standard, *d,l*-methylphenidate-d10, were purchased from Lipomed (Cambridge, MA). Certified reference standards lisdexamfetamine dimesylate and (±)-amphetamine and deuterated internal standard (ISTD), amphetamine-d11, were

purchased from Cerilliant Corporation (Round Rock, TX). Dibasic and monobasic phosphate solids used to prepare phosphate buffer (100 mM, pH 6) were purchased from Sigma Aldrich (St. Louis, MO). Deionized water was produced in-house using a Millipore Direct-Q® 3UV (Burlington, MA). Hexane, ethyl acetate, methanol and acetic acid were purchased from J.T Baker (Center Valley, MA). Ammonium hydroxide used during extraction was from Mallinckrodt Pharmaceuticals (St. Louis, MO). LC-MS grade acetonitrile and additive formic acid (>99.5%) used in mobile phase were purchased from Fisher Scientific (Hampton, NH). LC-MS grade ammonium formate was purchased from Millipore Sigma (Burlington, MA). Quantisal™ extraction buffer was acquired from Immunalysis Corporation (Pomona, CA).

Standard preparation

Standard preparation

Drug reference standards were prepared in methanol with the highest calibrator mix at 5000 ng/mL. An additional 8 calibrators were prepared via serial dilution at 1000, 500, 250, 100, 50, 20, 10 and 5 ng/mL, resulting in concentrations of 500, 100, 50, 25, 10, 5, 2, 1, 0.5 ng/mL when fortified in oral fluid. ISTD mix was prepared at 100 ng/mL in methanol, resulting in 10 ng/mL in oral fluid. For MPH, EPH and AMP, quality control (QC) mixes were prepared at concentrations of 1875, 375 and 75 ng/mL. When fortified in oral fluid, final concentrations were 75, 15, and 3 ng/mL. For LDX, QC mixes were prepared at concentrations of 6250, 1875, and 375 ng/mL. When fortified in oral fluid, final concentrations were 250, 75, and 15 ng/mL.

Extraction

For calibrators and controls, pooled oral fluid (250µL) was mixed with 3 parts Quantisal™ oral fluid buffer (total: 1mL) and fortified with the mixed working standards (25µL for calibrators or 10µL for QCs). All samples were fortified with ISTD mix (25µL), diluted with phosphate buffer (100mM, pH 6, 2mL), and loaded onto CEREX® Clin II SPE columns (Baldwin Park, CA) on a SPEWare System 48™ CEREX® Pressure Processor (Baldwin Park, CA). Columns were washed with 1mL deionized water, 1mL acetic acid (1M), dried under nitrogen, then washed with 1mL each of hexane, ethyl acetate and methanol. Analytes were eluted with 80:20 dichloromethane:iso-propyl alcohol with 5% ammonium hydroxide (1mL). Samples were dried under nitrogen (50ºC) using a Biotage TurboVap LV Evaporator (Charlotte, NC). Analytes were reconstituted in 100 µL of mobile phase (90:10 5mM ammonium formate and 0.01% formic acid in deionized water: 0.1% formic acid in acetonitrile) for analysis.

Instrumentation

Sample analysis was performed on an Agilent Technologies 1290 Infinity II liquid chromatograph coupled to an Agilent Technologies 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA). Analytes were separated using an Agilent Poroshell EC-C18 (2.1 x 100 mm, 2.7 µm with a matching guard. Separation was achieved using a gradient elution compromised of (A) 5mM ammonium formate and 0.01% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile. The LC method was a gradient elution with a starting mobile phase ratio of A:B 40:60 with an increase to 90%B within 8 minutes. The total run time was 10 minutes with an injection volume of 10 µL. Source conditions were positive mode electrospray ionization, drying gas temperature 350°C,

drying gas flow 13 L/min, nebulizer pressure 35 psi, sheath gas temperature 400°C, sheath gas flow 12 L/min, and capillary voltage 1500V. Analytes were detected with multiple reaction monitoring (MRM) with one transition for quantification and one transition for qualification per compound (Table 3.1).

Table 3.1. Optimized mass spectral parameters for cognitive stimulant quantification

Analyte	Retention Time (min)	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)	Collision Energy (V)	Fragmentor Voltage (V)	Internal Standard
Methylphenidate	2.94	234.1	84.1	56	21	102	MPH-d10
Ethylphenidate	3.43	248.2	84.1	56	21	107	MPH-d10
Amphetamine	1.93	136.1	119	91	5	61	AMP-d11
Lisdexamfetamine	1.52	264.2	84.1	56	25	107	AMP-d11
Methylphenidate-d10	2.89	244.2	93.1	61.1	25	97	-
Amphetamine-d11	1.89	147.2	130.1	70.0	5	61	-

Method validation

The quantitative method was validated using ANSI/ASB Standard 036: Standard Practices for Method Validation in Forensic Toxicology (25) as a guideline to address the following parameters: calibration model, limit of detection (LOD), lower limit of quantitation (LLOQ), precision, bias, ionization suppression/enhancement, processed sample stability, carryover, interference and dilution integrity.

Calibration models were determined using 8 non-zero calibrators (6 non-zero calibrators for LDX) fortified in five different sources of oral fluid over five days. Coefficients of determination values (R^2) were considered acceptable if R^2>0.99. Bias and precision (both within-run and between-run) were evaluated over three runs at three concentration levels (low: LQC, medium: MQC and high: HQC quality controls) in triplicate. Bias and precision were considered acceptable within ±20%.

Limit of detection (LOD) was determined in three different sources of oral fluid over three days. Analytes were assessed by peak shape, retention time reproducibility, ion ratios (±20%), and signal-to-noise ratios (S/N >3). Lower limit of quantitation (LLOQ) was assessed with the same guidelines as LOD with the addition of acceptable bias and precision (±20%bias or %CV, respectively) and S/N>10.

Ionization suppression/enhancement was evaluated using post-extraction addition of LQC and HQC in 10 different sources of blank oral fluid. Matrix effects (%) were calculated by comparison of analyte peak area in the post extraction samples to peak area in neat samples. Acceptable matrix effects were within ±25%. Ion enhancement and suppression are indicated by positive and negative results, respectively.

Processed sample stability was analyzed by reinjection LQC and HQC (n=3) extracts that were stored in the autosampler (4ºC) for 48 hours. Stability was determined by comparison of fresh (t_0) and processed (t_{48}) concentrations. Analytes were considered stable with acceptable bias (±20%).

Carryover was determined by comparison of analyte signal in the reinjection of a blank samples following the injection of the highest calibrator to the signal in the LOQ. Carryover was negligible if the reinject signal was <10% of the LOQ. Blank and negative (blank fortified with ISTD) oral fluid sources (n=5) were analyzed for target analytes to assess matrix and stable isotope interferences. A high concentration sample (without ISTD) was analyzed for deuterated compounds to ensure that non-deuterated analytes did not interfere with ISTD components. When determining interferences of commonly encountered drugs, four mixes of basic, neutral, and acidic drugs at 0.1 mg/mL in oral fluid were fortified into LQC samples. Compounds included in the interference mixes were Δ^9-tetrahydrocannabinol, alprazolam, amobarbital, amitriptyline, butalbital, caffeine, carbamazepine, carisoprodol, cocaine, codeine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, diphenhydramine, hydrocodone, hydromorphone, ketamine, methadone, nicotine, nordiazepam, oxazepam, oxycodone, pentobarbital, phencyclidine, phenobarbital, propoxyphene, secobarbital, tetrahydrocannabinolic acid, tramadol, and zolpidem. Accurate quantification of target analytes in LQC (±20%) indicate no quantitative interferences. For any sample that exceeded the working range, dilution integrity was assessed at a factor of 1:10. Dilution integrity was considered acceptable with bias values within ±20%.

Authentic samples

For proof of applicability, oral fluid samples (n=4) were anonymously collected from students at Sam Houston State University (Huntsville, TX) with Quantisal® devices following written informed consent (SHSU Protocol# IRB-2019-225). Before collection, subjects provided self-reported use of cognitive stimulant medication use and time of last use. All Quantisal devices turned blue within 5 min. The blue marker indicated that a sufficient amount of OF had been collected for analysis. After collection, samples were stored under refrigerated conditions (4°C), extracted and quantified using the methods above.

Results and Discussion

Method validation

Calibration models for MPH and EPH were quadratic (1/x weighting) and achieved R^2 values of >0.995 and >0.997, respectively. Calibration models for AMP and LDX were quadratic (1/x weighting) and achieved R^2 values of >0.998 and >0.999, respectively. For MPH, EPH and AMP, LOD and LOQ were 0.25 and 0.5 ng/mL, respectively. Linear ranges were 0.5-100 ng/mL for these analytes. For LDX, LOD and LLOQ were both 5 ng/mL with a linear range of 5-500 ng/mL. These data are summarized in Table 3.2. The calibration ranges were sufficient to encompass concentrations found in literature (16-18, 21). Though Comiran et al. detected *d*-AMP at a higher concentration than the current study (131.6 ng/mL) (21), dilution integrity was sustained at a factor of 1:10 in HQC with bias within ±7.3% for all analytes that may fall out of the calibration range. LOD and LLOQ values were comparable to those found in

literature (17, 19-21) and were determined to be acceptable for this method at 0.25 and 0.5 ng/mL, respectively (with the exception of LDX at 5 ng/mL for both LOD and LOQ).

Table 3.2. Calibration parameters, LOD, and LOQ for stimulants in oral fluid

Analyte	LOD (ng/mL)	LLOQ (ng/mL)	Calibration range (ng/mL)	Weighting (curve fit)	Mean R^2 (n=5)
Methylphenidate	0.25	0.5	0.5-100	1/x (linear)	0.995
Ethylphenidate	0.25	0.5	0.5-100	1/x (linear)	0.997
Amphetamine	0.25	0.5	0.5-100	1/x (quadratic)	0.998
Lisdexamfetamine	5	5	5-500	1/x (quadratic)	0.999

Abbreviations: limit of detection (LOD), lower limit of quantitation (LLOQ)

Bias ranged from -11.0 to -1.4% for all analytes at all three concentrations. Incorporating all concentration levels, between run precision ranged from 6.1 to 12.2 %CV for all analytes. Maximum within-run precision was 14.5, 15.7 and 14.0 %CV for LQC, MQC, and HQC, respectively. All bias and precision data were considered acceptable as all fell within ±20 %bias and %CV, respectively. The data for precision and bias are summarized in Table 3.3.

Table 3.3. Precision and bias validation results for stimulants in oral fluid

	Bias (%, n=15)			Between-run precision (%CV, n=15)			Maximum within-run precision (%CV, n=5)		
Analyte	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Methylphenidate	-1.4	-6.7	-7.3	8.7	6.0	7.9	3.7	2.5	14.0
Ethylphenidate	-7.2	-11.0	-6.2	8.6	5.3	10.4	12.6	4.7	14.0
Amphetamine	6.1	2.7	-7.7	8.0	10.3	12.2	14.5	15.7	6.2
Lisdexamfetamine	-7.3	-7.7	-2.6	6.1	7.9	10.1	8.7	6.8	13.7

Abbreviations: low quality control (LQC): 3 ng/mL (except LDX: 15 ng/mL), medium quality control (MQC): 15 ng/mL (except LDX: 75 ng/mL), high quality control (HQC): 75 ng/mL (except LDX: 250 ng/mL)
Replicates of LLOQ were n=9 for bias and between-run precision

Ion suppression/enhancement data were analyzed at two concentrations and are summarized in Table 3.4. At the low concentration, ion suppression was observed for all analytes (except LDX) at a range of -95.3 to -37.3%. LDX displayed ion enhancement at 40.9%. At the high concentration, MPH, EPH and MPH-d10 displayed ion suppression at -15.3, -86.4 and -14.9%, respectively. Ion enhancement was observed for AMP, LDX, and AMP-d11 at 14.9, 101.3 and 16.6%, respectively. Though there was ion suppression/enhancement observed outside the acceptable range (±25%), target analytes had comparable matrix effects with matched deuterated ISTD at both concentrations. Additionally, LOD values were not affected and matrix effects were reproducible in ten sources of oral fluid (0.3 to 14.2%CV in LQC and 3.5 to 11.3 %CV in HQC). To solve the solution of increased matrix effects, Immunalysis, which manufactures the Quantisal™ devices, suggests performing a sample clean up in oral fluid. This may help to prevent interference from the extraction buffer that is present (14). This phenomenon has been seen in additional studies which describe the potential for increased interferences from buffers when performing LC-MS/MS analysis (14).

Table 3.4. Matrix Effects (%) for stimulants in oral fluid

Analyte	LQC (n=10)	%CV	HQC (n=10)	%CV
Methylphenidate	-48.1[a]	3.3	-15.3	7.8
Ethylphenidate	-95.3[a]	1.4	-86.4[a]	8.2
Amphetamine	-46.7[a]	14.2	14.9	8.4
Lisdexamfetamine	40.9[a]	8.4	101.3[a]	3.5
Methylphenidate-d10	-49.9[a]	0.3	-14.9	11.3
Amphetamine-d11	-37.3[a]	13.5	16.6	11.0

Abbreviations: low quality control (LQC): 3 ng/mL (except LDX: 15 ng/mL), high quality control (HQC): 75 ng/mL (except LDX: 250 ng/mL)
[a]ion enhancement/suppression exceeding acceptable range (±25%)

All analytes were considered stable in the autosampler (4°C, 48 hours) with bias ranging from -13.2 to 8.7% and -17.0 to -9.1% in the LQC and HQC, respectively. No

carryover or interferences (qualitative or quantitative) were observed in blank, negative or fortified samples.

Authentic sample analysis

Summary of the results are presented in Table 3.5. Analytical findings support self-reported medication use. For samples 2-4, all subjects took a medication with amphetamine as the active ingredient and concentrations ranged from 6.0-28.6 ng/mL. Sample 1 was positive for lisdexamfetamine (5.8 ng/mL) and its' metabolite amphetamine at 78.8 ng/mL. There has been extensive research done on the detection and pharmacokinetics of lisdexamfetamine and amphetamine in plasma (26-40) but Comiran et al. has studied LDX pharmacokinetics in oral fluid. After taking a single dose of Vyvanse® (70 mg), oral fluid, plasma and urine were collected and analyzed for detection and quantification of LDX and AMP analytes. In the one-compartmental analysis performed, their study showed that LDX reached a maximum concentration of 8.27 ng/mL (1.5 hours post-administration) while *d*-AMP reached a maximum concentration of 286.76 ng/mL (4.2 hours post-administration) (22). Detection for LDX began at 1 hour for the subjects but detection time is short (5 hours). AMP was detected in OF as early as 0.25 hours with a half-life of 11 hours. Due to the pH difference between plasma and saliva, basic compounds get trapped in saliva and compounds like AMP accumulate which increase the concentration of this analyte in OF (22). Though the current study did not analyze the PD/PK of these analytes, the values reported are consistent to those found in literature with LDX being at a lower concentration than AMP. MPH was not detected in any of the samples, but this method is suitable to detect MPH at concentrations similar

to those found in literature. A blank, LOQ and authentic sample extracted ion chromatogram are displayed in Figure 3.1.

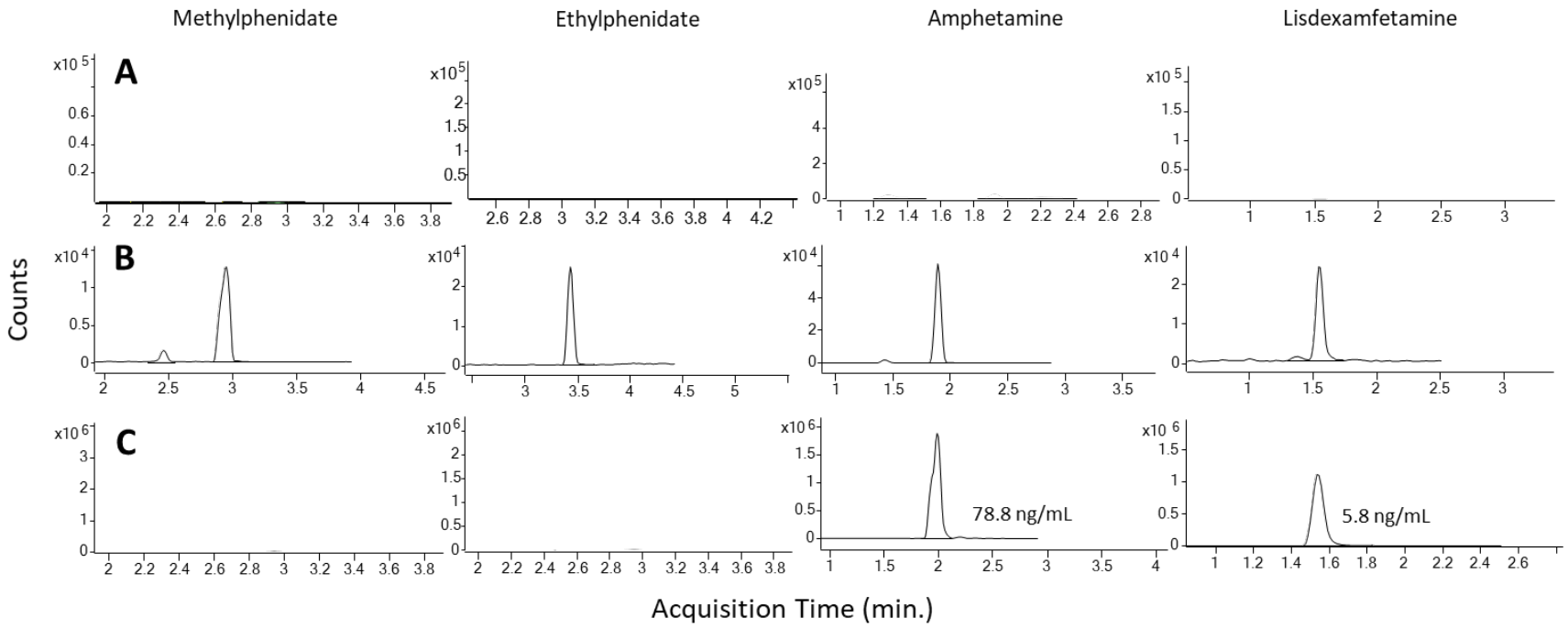

Figure 3.1. Extracted Ion Chromatogram for a blank (A), LOQ (B) and authentic sample (C) for all analytes

In this study, based off the self-reports, two students were non-compliant. Subject 2 reported taking more Adzenys than prescribed and Subject 3 took lower doses and less frequently than prescribed. However, detected concentrations appear within expected concentrations from single-dose clinical studies assessing cognitive stimulants in oral fluid. This method can be used in future studies to implicate potential abuse of these drugs on college campuses. This method provides the potential for assessing ADHD medication abuse and misuse in college populations through positive detection of non-prescribed medications paired with self-report surveys. Additionally, this analytical method was used to detect LDX and AMP in oral fluid proving it to be a useful matrix in the analysis of drugs of abuse in a forensic toxicology setting.

Table 3.5. Summary of authentic oral fluid sample data

Sample Number	Medication Ingested	Analyte detected	Concentration (ng/mL)
1	Vyvanse	Lisdexamfetamine	5.8
		Amphetamine	78.8
2	Adzenys	Amphetamine	28.6
3	Adderall	Amphetamine	6.0
4	Mydayis	Amphetamine	24.2

Conclusion

Oral fluid proves to be a promising alternative matrix for forensic toxicology as collection is quick and non-invasive. Additionally, oral fluid samples can be used in a variety of clinical and forensic settings. With the recent rise in cognitive stimulant abuse, it is important to develop methods to detect such stimulant drugs of abuse in order to provide interpretive value and assess drug compliance versus recreational use. This is the first method, to the author's knowledge, that presents a quantitative method for multiple

cognitive stimulants in oral fluid. This method was fully validated and verified by analysis of samples collected from college students.

References

CHAPTER IV

Chiral Separation and Quantitation of Methylphenidate, Ethylphenidate and Ritalinic Acid in Blood using Supercritical Fluid Chromatography[3]

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

[3]Smith CR, Vikingsson S, Kronstrand R, Swortwood, MJ. Chiral Separation and Quantitation of Methylphenidate, Ethylphenidate, and Ritalinic Acid in Blood using Supercritical Fluid Chromatography (2021), as submitted to *Drug Testing and Analysi*

Abstract

Supercritical fluid chromatography is a technique that analyzes temperature labile compounds, those with moderately low weight as well as chiral compounds. Methylphenidate (MPH) is a chiral compound with two chiral centers. MPH has two metabolites, ethylphenidate (EPH) and ritalinic acid (RA) which are also both chiral compounds. MPH is sold as a racemic mixture. The *d*- enantiomer of *threo*-MPH is responsible for medicinal effects. Due to the differing effects of the enantiomers, it is important to analyze the enantiomers individually to better understand their effect on the body. This method utilizes SFC and solid-phase extraction to separate and analyze the enantiomers of MPH, EPH and RA in postmortem blood. The linear range for MPH and EPH was 0.25-25 ng/mL and 10-1000 ng/mL for RA in blood. Bias ranged from -8.6 to -1.8% and precision was within 15.4% for all analytes. Following method validation, this technique was applied to the analysis of 49 authentic samples previously analyzed with an achiral method. Quantitative results for RA were comparable to achiral technique while there was loss of MPH and EPH over time. The *l:d* enantiomer ratio was calculated and MPH demonstrated greater abundance of the *d* enantiomer. Lastly, a short-term stability study was performed, indicating MPH instability over time. This is the first known method to separate and quantify the enantiomers of all three analytes utilizing SFC and SPE.

KEY WORDS: Cognitive stimulants, Supercritical fluid chromatography, Chiral separation

Introduction

Supercritical fluid chromatography is a type of chromatography that utilizes a supercritical fluid in the mobile phase. A supercritical fluid is a substance that is maintained above both its critical temperature and pressure (1). At this critical point, these substances display chemical properties of both a liquid and a gas (2, 3), as a gaseous substance can be used for separation like gas chromatography (GC) but can withstand high pressures seen in liquid chromatography (LC). Carbon dioxide is a common supercritical fluid selected due to its high speed, efficiency, low viscosity, and high diffusivity (1-4). Studies have demonstrated that this type of chromatography is good for analysis of low to moderate weight compounds, temperature labile compounds, and chiral compounds (1, 3). For a better understanding of SFC, there are several reviews that go into the theory and applications (2, 5-9).

Ritalin (methylphenidate) is a cognitive stimulating drug that is used to treat attention-deficit hyperactivity disorder (ADHD), a neural brain disease characterized by boredom, and difficulty hearing and listening (10). The lack of dopamine in the brain leads to inattention, hyperactivity and impulsivity. Methylphenidate (MPH) combats ADHD by blocking dopamine reuptake centers which increase dopamine in the synapse and speeds up brain activity (10-12). Structurally, MPH has two chiral centers. These two centers give rise to four stereoisomers: MPH: *erythro* [(*d*-(2R:2'R) and *l*-(2S:2'S)] and *threo* [[(*d*-(2R:2'R) and *l*-(2S:2'S)] (13). *Threo*-MPH has been shown to be responsible for the therapeutic effects of this medication, specifically the *d*- enantiomer while *erythro*-MPH produces toxic effects (10, 11, 14, 15). However, this medication is typically sold as a racemic mixture of *threo*-MPH with both enantiomers present. When

MPH is metabolized by the CES1 gene in the liver, its inactive metabolite ritalinic acid (RA) is produced (10, 16). In the presence of ethanol, MPH undergoes transesterification to produce ethylphenidate (EPH) (17, 18). Both metabolites also contain chiral centers which give rise to stereoisomers for these compounds as well. In one study, EPH was found at concentrations of 1 ng/mL and 8 ng/mL in whole blood in two suicide cases who had overdosed due to MPH and alcohol co-ingestion (19). Lately, studies have also shown that EPH can be purchased over the internet for recreational use (20, 21).

Studies have investigated the metabolism of MPH, EPH and RA drugs and the differing effects of these enantiomers (18, 22-25). With the enantiomers acting differently within the body, additional studies are needed to understand the pharmacokinetics and pharmacodynamics of these analytes. Due to prevalence in both medical and recreational settings, separation and analysis of these enantiomers needs to be investigated. Very few chiral methods exist to detect MPH enantiomers. Of those, only one study determined the MPH enantiomers and the metabolite, RA, in whole blood. This was the only case that utilized a solid-phase extraction (SPE) with a human biological matrix (10). Other chiral methods have determined the enantiomers of MPH (11, 14, 16) and EPH (16) utilizing liquid-liquid extraction from plasma (11, 16). Another chiral technique was developed utilizing SPE; however, the matrix of interest was mouse brain tissue (14). There are no studies that analyze all three analytes enantioselectively using blood. Though SFC has been used to analyze chiral compounds, it has not been used for MPH, EPH and RA.

Due to a lack of comprehensive techniques in the literature, this study sought to develop, optimize, and validate a method for the extraction, separation, and quantification of *d,l*-MPH, *d,l*-EPH, and *d,l*-RA from whole blood using SPE and SFC. As proof of

concept, the quantitative results from the chiral technique in this study were applied to 49 postmortem samples and the quantification values were compared to those obtained by a previously validated method that analyzed these same analytes without enantiomeric separation (26).

Materials and Methods

Chemicals and reagents

Methanolic *d,l-erythro*-methylphenidate, *d,l-erythro*-ethylphenidate, *d,l-erythro*-ritalinic acid and internal standards, *d,l*-d9-*threo*-methylphenidate and *d,l*-d10-*threo*-ritalinic acid, were purchased from Cerilliant (Round Rock, TX, USA). The pure enantiomers *d-threo*- methylphenidate and *l-threo*-methylphenidate were obtained from Sigma Aldrich (St Louis, MI, USA). Bond Elut Ceritfy SPE columns (130 mg, 10 mL cartridge, 120 µm particle size) were purchased from Agilent Technologies (Santa Clara, CA, USA). Methanol, 2-propanol, potassium dihydrogen phosphate, potassium hydroxide, glacial acetic acid, and formic acid were purchased from Merck (Darmstadt, Germany). Purified water came from a Milli-Q water system by Millipore Sigma (Burlington, MA, USA).

Preparation of standard solutions

A stock solution of each standard was prepared at 10,000 ng/mL in methanol. Mixed methanolic solutions for calibrators were prepared via serial dilution. When fortified in blood, the following concentrations of the combined racemate were produced: 0.25, 0.75, 2.5, 7.5, and 25 ng/mL for MPH and EPH and 10, 30, 100, 300, 1000 ng/mL for RA. Quality controls were prepared independently via serial dilution. When fortified in blood, the quality control concentrations were: 0.5 ng/mL (low) and 20 ng/mL(high)

for MPH and EPH and 20 ng/mL (low) and 800 ng/mL (high) for RA. A methanolic internal standard (ISTD) solution was prepared at 1000 ng/mL which resulted in a concentration of 100 ng/mL when fortified in blood. All solutions were stored in amber vials at -20°C.

Solid-phase extraction

Blood (250 µL) was fortified with 25 µL of calibrator or QC solution. Calibrators, quality controls, negative controls, and case samples were fortified with 25 µL of ISTD solution. Phosphate buffer (100 mM, pH 6.0, 1 mL) was added to all samples and vortexed to mix. The SPE columns (non-polar C8 sorbent and strong cation-exchange sorbent) were pre-conditioned with methanol (1 mL) and phosphate buffer (1 mL). Samples were centrifuged (2000 rpm, 10 min) before loading onto the SPE column. Columns were washed with 1mL each of acetic acid (0.1M) and methanol. Analytes were eluted with 2% ammonium hydroxide in methanol (2 mL). The eluates were evaporated to dryness under nitrogen and reconstituted in 0.2% trifluoroacetic acid (TFAA) in methanol (100µL).

Instrumentation

Supercritical fluid chromatography/liquid chromatography

Analysis was performed on an Agilent 1260 Infinity SFC Control Module coupled to an Agilent 1260 Infinity II liquid chromatograph. The SFC back-pressure regulator was held at 60°C and 120bar. Analytes were separated on an Agilent Poroshell Chiral-V column (2.1x100mm, 2.7µm). The column temperature was held at 20°C. Separation was achieved with mobile phase utilizing supercritical CO_2 (A) and 0.2% TFAA in methanol (B) at a flow rate of 1.8mL/min. Separation was achieved using

gradient elution starting at 75:25 (A:B) for 4.5min. Mobile phase B was increased to 40% over 0.75 min at a flow rate of 1.35 mL/min. These conditions were held for 0.75 min. Mobile phase B was then reduced to 12% over 0.2 min and the flow rate was increased to 1.8 mL/min for an additional 1.5 min. The total run time was 7.7 min. A solution of methanol:deionized water (diH_20) (85:15) with 0.1% formic acid was mixed with column eluent at a flow rate of 0.3 mL/min prior to entering ESI chamber. A total of 3 µL was injected onto the SFC-MS for analysis.

Mass spectrometry

Detection of the analytes was performed on an Agilent Ultivo Triple Quadrupole. Electrospray ionization was operated in positive mode. Multiple reaction monitoring (MRM) was used to detect the analytes. One transition for quantification and one transition for qualification was used. Data acquisition and analysis were performed using Agilent MassHunter Workstation (Santa Clara, CA, US). The gas temperature was at set at 175°C with a gas flow of 13 L/min. The nebulizer was at 20 psi. The sheath gas temperature was at 300°C with a flow of 12 L/min. The nozzle voltage was set at 2000 V and the capillary voltage was at 6000 V.

Method validation

A fit-for-purpose validation in blood was carried out following international guidelines (27). The validation parameters evaluated included calibration model, linearity, bias, precision, matrix effects, and recovery.

Calibration models were evaluated using 5 non-zero calibrators over 5 days. Linearity was determined using the least squares model and an R^2>0.99 was considered acceptable.

Bias and precision were determined at two different QC concentrations in triplicate over 5 days. The low QC was 0.5 ng/mL (MPH and EPH) and 20 ng/mL (RA). The high QC was 20 ng/mL (MPH and EPH) and 800 ng/mL (RA). Bias (%) and precision (coefficient of variation, %CV) were considered acceptable within ±20%. Within-run and between-run precision were calculated for both QC concentrations.

Recovery was determined by taking the mean response of the extracted samples compared to the mean response of the post-extraction fortified samples. Matrix effects were determined by taking the mean response of post-extraction fortified samples compared to the mean response of neat standards. Matrix effects were considered acceptable when they fell within ±25%. Five different sources of blood were used for these experiments. Recovery and matrix effects were assessed at 500 ng/mL.

Analysis of authentic samples

Authentic samples were included under the approval of the regional ethics committee in Linköping (Dnr: 2018–186/31). A total of 49 blood samples were collected that had reported methylphenidate, ethylphenidate or ritalinic acid concentrations from an achiral routine method at National Board of Forensic Medicine in Linköping, Sweden (26) with limit of quantitation of 0.2 ng/g and 10 ng/g for MPH and RA, respectively. The samples were originally analyzed using the achiral method between January 2019 and November 2019 and reanalyzed at different times after the original analysis.

Results and Discussions

Method development

The shortage of previously published methodology for chiral separation using SFC prompted some method development to ensure high sensitivity and adequate enantioselective separation. The Agilent Poroshell Chiral-V column and Phenomenex Lux Amylose column were utilized to try and obtain the best resolution. Separation was investigated using methanol or acetonitrile as mobile phase B. Varying percentages (0.1-0.5%) of trifluoroacetic acid, triethylamine, and ammonium formate were used as additives. Several gradients were evaluated with varying starting compositions to achieve optimal resolution of enantiomers for all 6 analytes. The Chiral-V column and the gradient described in 2.4.1 resulted in the best enantiomeric separation while keeping column and instrument pressures to a minimum. Chromatograms from an extracted calibrator are shown in Figure 4.1. Full baseline resolution was obtained for the enantiomers of MPH and EPH and near baseline resolution was obtained for RA. Injection volume was also optimized for this method (1- 7µL) with consideration to peak shape, peak area, and separation. An injection volume of 3 µL enabled the quantification of 0.25 ng/mL for MPH and EPH with sustained peak shape and separation. An individual standard for each enantiomer of MPH was obtained. These analytes were run as neat standards using the instrumentation as described above. These standards were used to determine the elution order of the *d* and *l* enantiomer of MPH. As previous literature has shown, the *l* enantiomer eluted first followed by the *d* enantiomer (10).

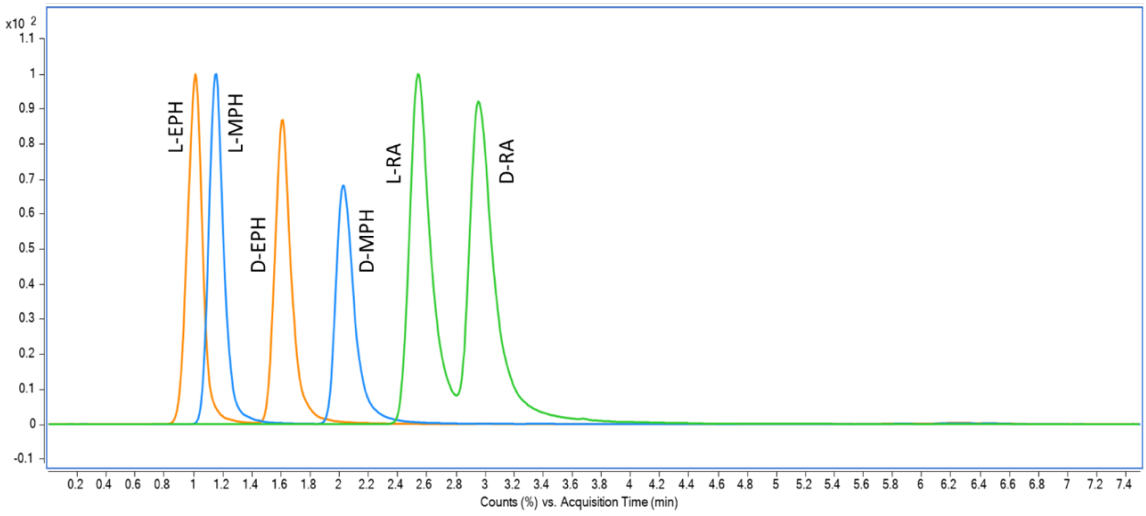

Figure 4.1. Final optimized chromatography of all analytes

Method validation

Calibration curves were constructed with 1/x weighting and resulted in R^2 values ≥ 0.99. The linear range for MPH and EPH was 0.25-25 ng/mL and 10-1000 ng/mL for RA. Bias was -8.6 to -1.8% for all analytes. Maximum within-run and between-run precision was 15.4% and 12.0%, respectively. All of these met validation criteria and were considered acceptable. The lowest calibrator was considered the lower limit of quantification. The LQC and HQC were 0.5 and 20 ng/mL for *d,l*-MPH and *d.l*-EPH, respectively. For RA, The LQC and HQC were 20 and 800 ng/mL, respectively. Analyte recovery was >80% for all analytes. Matrix effects were -5.2 to -10.8% for MPH and EPH. The *d*-enantiomer of RA was the only analyte that fell out of the acceptable ±25% range with 26% ion suppression. However, its matched deuterated internal standard exhibited similar matrix effects and therefore was deemed acceptable. These results are summarized in Table 4.1.

Table 4.1. Linear range, bias, precision (within-run and between run), and matrix effects of all analytes

Analyte (Matched ISTD)	Linear Range (ng/mL)	Bias (%)		Within-run precision (Max, %)		Between run precision (%)		Matrix effects % (IS %)
		LQC	HQC	LQC	HQC	LQC	HQC	
l-RA (d10-*l*RA)	10-1000	-7.4	-0.8	15.2	6.9	11.4	7.9	-24.3 (-6.6)
d-RA (d10-*d*RA)	10-1000	-8.6	-1.7	15.4	11.0	12.1	8.6	-26.5 (-13.8)
l-MPH (d9-*l*MPH)	0.25-25	-6.8	-4.3	12.0	8.0	9.8	7.8	-10.8 (5.8)
d-MPH (d9-*d*MPH)	0.25-25	-4.7	-1.4	12.5	6.6	11.6	9.8	-7.6 (11.6)
l-EPH	0.25-25	-3.7	-3.1	14.1	10.2	10.9	7.8	-7.6
d-EPH	0.25-25	-1.8	0.8	13.2	7.0	10.4	9.1	-5.2

Authentic samples

Authentic case samples (n=49) that were previously analyzed by an achiral method (26) were selected for quantitative analysis. The quantitative values for each enantiomer are summarized in Table 4.2.

Table 4.2. Summary of the number of positive cases in postmortem blood with the quantitative value of each enantiomer from the chiral analysis

Analyte	Positive Cases	Mean (Range) Concentration (ng/mL)
l-RA	49	343 (<LOQ-3419)
d-RA	49	436 (17-5410)
l-MPH	15	6 (<LOQ-20.5)
d-MPH	29	28.5 (<LOQ-358.2)
l-EPH	5	2.5 (0.62-8.2)
d-EPH	1	<LOQ

From the table it can be noted that both enantiomers of RA were present in all the cases. At least one enantiomer of MPH was present in 29 cases while at least one

enantiomer of EPH was present in 5 cases. All 5 cases had ethanol present in blood suggesting that EPH may be a result of alcohol co-administration rather than originating from an intake of EPH (28). The quantitative values from the initial achiral analysis are summarized in Table 4.3.

Table 4.3. Summary of the number of positive cases with the quantitative value of each enantiomer from the achiral analysis

Analyte	Positive Cases	Mean (Range) Concentration (ng/mL)
RA	49	805 (29-5900)
MPH	37	35 (0.6-400)
EPH	7	3 (0.5-10)

Percent difference was calculated between the two methods using total quantitative values (*l*+*d*) from the chiral results compared to those initially obtained with the achiral technique. When comparing the two techniques, the quantitative results were comparable for RA with an average of 7.3% difference and a range between -40% and +28%. For MPH 12 cases that originally had low, but quantifiable concentrations were negative with the chiral method. The range in difference was –82% to +17% with a mean value of -33% and a possible explanation being instability of MPH. Smith et al. conducted a short-term stability study to assess the degradation of MPH at multiple conditions. MPH metabolized to RA within one week and 48 hours at room temperature and elevated temperatures, respectively, suggesting instability (29).

The cases had storage times between 1 and 11 months. In Figure 4.2, the percent difference between the analyses of MPH is depicted as a function of storage time. There was a negative correlation suggesting that time is a relevant factor even though samples could be stored for up to three months before showing greater losses of MPH.

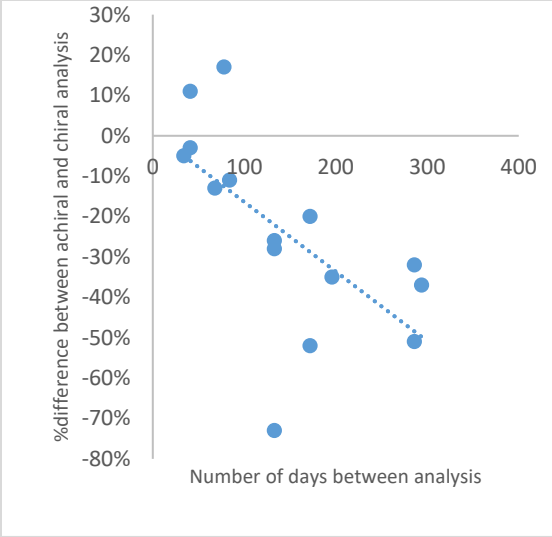

Figure 4.2. Percent difference of the achiral and chiral analysis of MPH as a function of time

Next, the *l:d* ratio was calculated using the samples in which both enantiomers were present. The results of this analysis are summarized in Table 4.4. In most cases, methylphenidate is sold as a racemic mixture of *threo*-MPH. This study found the *d* enantiomer to be in higher abundance than the *l* enantiomer for MPH accounting for 84% of the total concentration of MPH. This may be due to preferential metabolism of the *l* enantiomer (23). However, for RA, the *d* and *l* enantiomers were similar in concentrations. When comparing the total concentration values, RA was found to be approximately 5x or higher in authentic blood samples as compared to MPH. This work is consistent with that of Thomsen and colleagues who found RA to be in higher abundance than MPH (10). Time between drug intake and sampling/death may play a role in the enantiomeric difference of *l* and *d* ratios but metabolism studies need to be performed in the future to understand the differences of these enantiomers in blood.

Table 4.4. Summary of the number of positive cases and the l/d ratio for the enantiomers of RA and MPH

Analyte	Number of cases	*l/d* ratio Mean (Range)
l-RA	49	0.68 (0.07-1.84)
d-RA		
l-MPH	15	0.14 (<0-0.38)
d-MPH		

Conclusions

We have, for the first time, explored SFC to separate and quantify the enantiomers of MPH, EPH and RA in blood. We conclude that SFC-MS/MS successfully separated the enantiomers of MPH, its major metabolite RA as well as the minor metabolite EPH with a comparatively short chromatography. Full baseline resolution was achieved for the enantiomers of MPH and EPH and near baseline separation for RA enantiomers. In addition, the quantification of the analytes showed good accuracy and acceptable precision. The linear ranges covered the concentrations typically found, although some samples required dilution for accurate RA quantification. The SFC-MS/MS method provides some insight into the prevalence of *d* and *l* enantiomers in biological samples and the possible instability of these compounds. Methylphenidate is sold as a racemic mixture of *threo*-MPH enantiomers. However, in blood we found that *d*-MPH accounted for 84% of the total concentration of MPH. Our lab is currently investigating this breakdown as part of a long-term stability study of these analytes.

References

1. Radcliffe, C., Maguire, K. and Lockwood, B. (2000) Applications of supercritical fluid extraction and chromatography in forensic science. Journal of Biochemical and Biophysical Methods, 43, 261-272.
2. Tarafder, A. (2016) Metamorphosis of supercritical fluid chromatography to SFC: An Overview. TrAC Trends in Analytical Chemistry, 81, 3-10.
3. West, C. (2019) Recent trends in chiral supercritical fluid chromatography. TrAC Trends in Analytical Chemistry, 120, 115648.
4. Sie, S.T., Van Beersum, W. and Rijnders, G.W.A. (1966) High-Pressure Gas Chromatography and Chromatography with Supercritical Fluids. I. The Effect of Pressure on Partition Coefficients in Gas-Liquid Chromatography with Carbon Dioxide as a Carrier Gas. Separation Science, 1, 459-490.
5. Lesellier, E. and West, C. (2015) The many faces of packed column supercritical fluid chromatography – A critical review. Journal of Chromatography A, 1382, 2-46.
6. Saito, M. (2013) History of supercritical fluid chromatography: Instrumental development. Journal of Bioscience and Bioengineering, 115, 590-599.
7. Nováková, L., Grand-Guillaume Perrenoud, A., Francois, I., West, C., Lesellier, E. and Guillarme, D. (2014) Modern analytical supercritical fluid chromatography using columns packed with sub-2μm particles: A tutorial. Analytica Chimica Acta, 824, 18-35.

8. Silva, M.R., Andrade, F.N., Fumes, B.H. and Lanças, F.M. (2015) Unified chromatography: Fundamentals, instrumentation and applications†. Journal of Separation Science, 38, 3071-3083.

9. Pauk, V. and Lemr, K. (2018) Forensic applications of supercritical fluid chromatography – mass spectrometry. Journal of Chromatography B, 1086, 184-196.

10. Thomsen, R., Rasmussen, H.B., Linnet, K. and the, I.C. (2012) Enantioselective Determination of Methylphenidate and Ritalinic Acid in Whole Blood from Forensic Cases Using Automated Solid-Phase Extraction and Liquid Chromatography–Tandem Mass Spectrometry. Journal of Analytical Toxicology, 36, 560-568.

11. Ramos, L., Bakhtiar, R., Majumdar, T., Hayes, M. and Tse, F. (1999) Liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry enantiomeric separation of dl-threo-methylphenidate, (Ritalin®) using a macrocyclic antibiotic as the chiral selector. Rapid Communications in Mass Spectrometry, 13, 2054-2062.

12. Ritz, M.C., Lamb, R.J., Goldberg, S.R. and Kuhar, M.J. (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science, 237, 1219-23.

13. Markowitz, J.S., Straughn, A.B. and Patrick, K.S. (2003) Advances in the Pharmacotherapy of Attention-Deficit-Hyperactivity Disorder: Focus on Methylphenidate Formulations. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 23, 1281-1299.

14. Combs Carolyn, C., Hankins Erin, L., Copeland Cara, L., Brown Stacy, D. and Pond Brooks, B. (2013) Quantitative determination of d- and l-threo enantiomers of methylphenidate in brain tissue by liquid chromatography–mass spectrometry. Biomedical Chromatography, 27, 1587-1589.

15. Szporny, L. and Görög, P. (1961) Investigations into the correlations between monoamine oxidase inhibition and other effects due to methylphenydate and its stereoisomers. Biochemical Pharmacology, 8, 263-268.

16. Zhu, H.-J., Patrick, K.S. and Markowitz, J.S. (2011) Enantiospecific determination of dl-methylphenidate and dl-ethylphenidate in plasma by liquid chromatography–tandem mass spectrometry: Application to human ethanol interactions. Journal of Chromatography B, 879, 783-788.

17. Patrick, K.S., Straughn, A.B., Reeves, O.T., Bernstein, H., Bell, G.H., Anderson, E.R., et al. (2013) Differential Influences of Ethanol on Early Exposure to Racemic Methylphenidate Compared with Dexmethylphenidate in Humans. Drug Metabolism and Disposition, 41, 197-205.

18. Koehm, M., Kauert, G.F. and Toennes, S.W. (2010) Influence of ethanol on the pharmacokinetics of methylphenidate's metabolites ritalinic acid and ethylphenidate. Arzneimittelforschung, 60, 238-44.

19. Markowitz, J., Logan, B., Diamond, F. and S. Patrick, K. (1999) Detection of the Novel Metabolite Ethylphenidate After Methylphenidate Overdose With Alcohol Coingestion. Journal of Clinical Psychopharmacology, 19, 362-366.

20. Krueger, J., Sachs, H., Musshoff, F., Dame, T., Schaeper, J., Schwerer, M., et al. (2014) First detection of ethylphenidate in human fatalities after ethylphenidate intake. Forensic Science International, 243, 126-129.

21. Casale, J.F. and Hays, P.A. (2011) Ethylphenidate: an analytical profile. Microgram Journal, 8, 58-61.

22. Srinivas, N.R., Hubbard, J.W., McKay, G., Hawes, E.M. and Midha, K.K. (1991) In vitro hydrolysis of RR,SS-threo-methylphenidate by blood esterases--differential and enantioselective interspecies variability. Chirality, 3, 99-103.

23. Srinivas, N.R., Hubbard, J.W., Korchinski, E.D. and Midha, K.K. (1993) Enantioselective Pharmacokinetics of dl-threo-Methylphenidate in Humans. Pharmaceutical Research, 10, 14-21.

24. Srinivas, N.R., Hubbard, J.W., Korchinski, E.D. and Midha, K.K. (1992) Stereoselective Urinary Pharmacokinetics of dl-threo-Methylphenidate and Its Major Metabolite in Humans. Journal of Pharmaceutical Sciences, 81, 747-749.

25. Patrick, K.S., Straughn, A.B., Minhinnett, R.R., Yeatts, S.D., Herrin, A.E., DeVane, C.L., et al. (2007) Influence of Ethanol and Gender on Methylphenidate Pharmacokinetics and Pharmacodynamics. Clinical Pharmacology and Therapeutics, 81, 346-353.

26. Josefsson, M. and Rydberg, I. (2011) Determination of methylphenidate and ritalinic acid in blood, plasma and oral fluid from adolescents and adults using protein precipitation and liquid chromatography tandem mass spectrometry—A method applied on clinical and forensic investigations. Journal of Pharmaceutical and Biomedical Analysis, 55, 1050-1059.

27. Peters, F.T., Drummer, O.H. and Musshoff, F. (2007) Validation of new methods. Forensic Sci Int, 165, 216-24.

28. Aoyama, T., Kotaki, H., Honda, Y. and Nakagawa, F. (1990) Kinetic analysis of enantiomers of threo-methylphenidate and its metabolite in two healthy subjects after oral administration as determined by a gas chromatographic-mass spectrometric method. Journal of pharmaceutical sciences, 79, 465-469.

29. Smith, C.R. and Swortwood, M.J. (2021) Short- and Long-Term Stability of Methylphenidate and Its Metabolites in Blood. Journal of Analytical Toxicology, 45, 863-869.

CHAPTER V

Short- and Long-Term Stability of Methylphenidate and its Metabolites in Blood[4]

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

[4]Smith CR and Swortwood MJ. (2021). Short- and Long-Term Stability of Methylphenidate and Its metabolites in Blood. *Journal of Analytical Toxicology,* **45,** 863-869

Abstract

Methylphenidate is a medication used to combat attention-deficit/hyperactivity disorder by speeding up brain activity. Methylphenidate has two chiral centers; however, *d-threo*-methylphenidate is responsible for its effects. Few studies have analyzed methylphenidate and its metabolites, ritalinic acid and ethylphenidate, in blood. Stability studies are crucial in a forensic setting to provide insight on ideal storage conditions and analysis time. In this study, *d,l*-methylphenidate, *d,l*-ethylphenidate and ritalinic acid were analyzed at two concentrations (15 and 150 ng/mL) over 5 months at room temperature (~25°C), refrigerated (4°C), frozen (-20°C), and elevated (35°C) temperatures. Analytes were analyzed using a validated liquid-chromatography mass spectrometry method. Ritalinic acid concentrations increased 53% at 25°C after 24h while *d*- and *l*-methylphenidate concentrations dropped 18.1% and 20.6%, respectively. Additionally, *d*- and *l*-ethylphenidate concentrations decreased 22.3% and 28.8%, respectively. All analytes were stable at 4°C for one week (±17% change). At -20°C, all analytes were stable for 5 months. At 35°C, *l*-ethylphenidate remained stable for 24h (14.4% loss) at the high concentration while ritalinic acid increased 244%. Losses of 64.1%, 68.7% and 27.2% were observed for *d*-methylphenidate, *l*-methylphenidate and *d*-ethylphenidate, respectively. Due to this, a follow up study was designed to assess the breakdown of methylphenidate. The short term experiment assessed *d,l*-methylphenidate at two concentrations for one month in the same conditions. As methylphenidate decreased, ritalinic acid concentrations rose. At 25°C, it took two weeks for methylphenidate to metabolize completely into ritalinic acid. In refrigerated and frozen

temperatures, methylphenidate did not completely metabolize to ritalinic acid. In elevated temperatures, methylphenidate broke down to ritalinic acid within two weeks. Due to this, it was concluded that *d,l*-methylphenidate breaks down in the blood to its metabolite ritalinic acid and may make data interpretation difficult if samples are not properly stored. The optimal storage for these analytes is recommended at -20°C.

KEY WORDS: Cognitive stimulants, Stability, Chiral Separation, LC-MS/MS

Introduction

Methylphenidate (MPH) is a cognitive stimulant used to treat attention-deficit/hyperactivity disorder. Due to its ability to inhibit dopamine reuptake, MPH allows for an increase in dopamine which leads to increased brain stimulation and improved focus (1-5). MPH is the most prescribed medication to combat this disorder (6, 7). Though it has medicinal uses, it also has a high potential for recreational abuse, making it applicable in a forensic setting (8). MPH contains two chiral centers, giving rise to four stereoisomers. The *d* enantiomer of the *threo*-isomer is responsible for the pharmaceutical effects of MPH (9-14). Due to the differing effects of the enantiomers, it is important to study the pharmacodynamic and pharmacokinetic properties of this drug (6, 12, 15). MPH breaks down into its inactive metabolite, ritalinic acid (RA) by CES1A1 in the liver (16-18). In the presence of alcohol, MPH will also produce ethylphenidate (EPH) (19). EPH was first identified 50 years ago as an internal standard for laboratory assays when studying methylphenidate. It was then later discovered that EPH is an active metabolite of MPH (20-22). EPH has displayed greater affinity for the dopamine transporter when compared to MPH making it have a high potential for addiction (23, 24). EPH has been reported to be sold online or in headshops under various street names such as “nopaine” (25, 26). Given its similarity of structure, EPH is expected to act the same was as MPH in the body, making it of forensic interest (27).

Stability is a significant aspect to consider in a forensic setting. It is important to understand the stability of drugs in various matrices so analysis can be performed in a timely fashion, to ensure accurate quantitative results and ultimately aid in data interpretation. Previous studies have assessed short-term stability of MPH alone and with

its metabolites in various matrices. One study found that MPH in plasma was stable for 5 freeze thaw cycles (if analysis is within 0.5h) (28). In this same study, MPH was stable in blood for 6h at room temperature and the derivatized extract of MPH was also stable at 4°C for 10 days (28). Zhu et al. found MPH to be stable in plasma on the benchtop at ambient temperature for 4h, through 3 freeze-thaw cycles and for 24h at 4°C in the autosampler (6). Wargin et al. found the half-life of MPH in plasma to be 42.76h (29) while Seçilir et al. noted the optimal storage condition is at -20°C after collection (30). Thomsen et al. examined MPH and RA stability in blood and found that analytes were stable on the benchtop for 6h, through two freeze-thaw cycles, and for 17 days at -20°C. Additionally, the extracts were stable at ambient temperature for 24 hours (15). Similarly, MPH and RA were analyzed in both blood and plasma and found to be stable for 3 months at -20°C and through 5 freeze-thaw cycles. After protein precipitation, MPH and RA were stable in the autosampler at 10°C for 3 days and at -20°C for one week. MPH and RA were found to be unstable when left at ambient temperature (31). Few studies investigated EPH as its stability is assumed to be comparable to MPH due to structural similarities (25, 32). In plasma, the enantiomers of EPH were found to be stable on the benchtop at ambient temperature for 4h, after 3 freeze-thaw cycles, and at 4°C in the autosampler for 24h (6). Though these studies have assessed stability of these analytes over a short period of time, to our knowledge, no studies have analyzed the stability of these analytes over a long period of time. This will be the first method to analyze the stability of RA, *d,l*-MPH and *d,l*-EPH using liquid chromatography-mass spectrometry (LC-MS/MS).

As limited long-term stability data exist, this study aimed to analyze *d,l*-MPH, *d,l*-EPH and RA in blood stored in four temperature conditions: room temperature (~25°C),

refrigerated (4°C), frozen (-20°C) and elevated temperature (35°C) for up to 5 months. In addition, separate experiments were designed to model MPH degradation to RA in fortified blood samples. To our knowledge, this is the only validated method in literature to analyze all 5 analytes in blood by LC-MS/MS.

Materials and Methods

Chemicals and reagents

Methanolic solutions (1 mg/mL) of *d,l*-methylphenidate and internal standards, *d,l-threo*-methylphenidate-d10 and *d,l-threo*-ritalinic acid-d10 and ethanolic solutions of *d,l-threo*-ethylphenidate and ritalinic acid were purchased from Lipomed (Cambridge, MA, US). Defibrinated bovine blood with sodium fluoride and potassium oxalate preservatives was purchased from Quad Five (Ryegate, MT, US) and stored at 4°C. DAU Clean-Screen (130 mg, 3 mL) columns (United Chemical Technologies, Bristol, PA, US) were utilized for solid-phase extraction (SPE) on a SPEWare System Pressure Processor. A Biotage TurboVap LV (Charlotte, NC, US) equipped with nitrogen gas was used for evaporation. For the mobile phase, trifluoroacetic acid (99.5%) and ammonium acetate (LC-MS Ultra) were purchased from Fisher Scientific (Hanover Park, IL, US). Acetic acid was purchased from Mallinckrodt Pharmaceuticals (St. Louis, MO, US) and LC-MS grade deionized water was purchased from Honeywell (Charlotte, NC, US). Ammonium hydroxide and LC-MS methanol (>99.9%) used in sample preparation, extraction and in the mobile phase were from J.T. Baker (Center Valley, MA, US). Dibasic sodium phosphate and monobasic sodium phosphate were from Sigma-Aldrich (St. Louis, MO, US). BD Vacutainer™ tubes (10mL, 16 x 100mm) without preservative were acquired from VWR (Radnor, PA, US).

Preparation of standards in blood

All analytes were prepared as stock standards in methanol at a concentration of 100,000 ng/mL. Mixed methanolic solutions were prepared via serial dilution, resulting in concentrations of 5, 10, 50, 100, 500, 1000, 2000, 5000 ng/mL. When fortified in blood, the following concentrations were produced: 0.5, 1, 5, 10, 50, 100, 200, 500 ng/mL. Quality controls (QC) were prepared separately in the same way as mixed methanolic solutions resulting in the following concentrations: 15 ng/mL (low), 250 ng/mL (medium) and 4000 ng/mL (high) for RA and 1500 ng/mL (high) for MPH and EPH. When fortified in blood, the following concentrations were produced: 1.5 ng/mL (low), 25 ng/mL (medium) and 400 ng/mL (high) for RA and 150 ng/mL (high) for MPH and EPH. A mixed methanolic internal standard solution (ISTD) of *d,l*-methylphenidate-d10 and *d,l*-ritalinic acid-d10 was prepared at 100 ng/mL, resulting in 10 ng/mL when fortified in blood. All solutions were stored at -20°C in amber vials.

For long-term stability, all analytes were prepared at 100,000 ng/mL in methanol. Blood (100mL) was fortified to a final concentration of 15 ng/mL or 150 ng/mL MPH, EPH, and RA to represent low quality control (LQC) or high quality control (HQC), respectively. Fortified blood aliquots (~5 mL) were distributed into vacutainer tubes and stored at: room temperature (~25°C), refrigerated (4°C), frozen (-20°C) and elevated temperature (35°C). Temperatures were monitored by thermometer or digital sensors. After preparation, samples from each tube were immediately analyzed (T_0) and then in triplicate after 24h, 48h, 72h, 1wk, 2wk, 3wk, 6wk, 2mo, 3mo, 4mo,and 5mo.

For short term stability, LQC and HQC blood was prepared with only MPH and then distributed into separate vacutainers at the same temperature settings. For this study, 8 time points were analyzed in duplicate (T_0, 24h, 48h, 72h, 1wk, 2wk, 3wk, 4wk).

Extraction

For quantification, blood (250 µL) was fortified with 25µL of calibrator or QC solution. All samples were fortified with 25 µL of ISTD solution, diluted with 1 mL phosphate buffer (100mM, pH6), and centrifuged (2000rpm, 10min). SPE columns were conditioned with methanol (1mL) and phosphate buffer (1mL) before the sample was loaded. The columns were washed with acetic acid (0.1M, 1mL) and methanol (1mL) before drying under nitrogen. Analytes were eluted with 2% ammonium hydroxide in methanol (2mL) and evaporated to dryness at 50°C. Samples were reconstituted in 100 µL of mobile phase and transferred to autosampler vials. A total of 1 µL was injected onto the LC-MS/MS.

Instrumentation

Liquid chromatography

Analysis was performed on an Agilent 1290 Infinity II Liquid Chromatograph coupled to an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA). An Agilent Poroshell Chiral-V column (2.7 µm, 2.1 x 100 mm) with a Poroshell 120 EC-C18 (2.7uL, 2.1 x 5 mm) guard was used for separation of analytes. Column temperature was held at 35°C. Analyte separation used an isocratic elution with mobile phase A:B at 2:98 at 0.6 mL/min. Aqueous mobile phase (A) was deionized water. Organic mobile phase (B) consisted of 0.0125% trifluoroacetic acid (v/v) and 0.025% ammonium acetate (w/v) in methanol. Total run time was 4 minutes.

Mass spectrometry

Electrospray ionization in positive mode was used. Multiple reaction monitoring (MRM) was used for analyte detection using with one transition each for quantification and qualification. The ion transitions and retention times are listed in Table 5.1. The gas temperature and gas flow were set at 300°C and 5 L/min, respectively. The sheath gas temperature and flow were 350°C and 11 L/min, respectively. The capillary voltage was 3500 V with the nebulizer at 45 psi. Data acquisition and analysis were performed using Agilent MassHunter Workstation software (Santa Clara, CA).

Table 5.1. Retention time and precursor ions with quantitative (top) and qualitative (bottom) product ions for all five analytes and three internal standards

Analyte	Retention time (min)	Precursor Ion *(m/z)*	Product Ion *(m/z)*
d-MPH	1.372	234.1	84.1
			56.0
l-MPH	1.104	234.1	84.1
			56.0
d-EPH	1.140	248.2	84.1
			56.0
l-EPH	0.994	248.2	84.1
			56.0
RA	0.656	220.1	84.0
			56.1
d-MPH-d10	1.409	244.2	93.1
			61.1
l-MPH-d10	1.126	244.2	93.1
			61.1
RA-d10	0.654	230.2	93.1
			61.1

Method validation

This study utilized a previously validated method that used the AAFS Standards Boards (ASB) Standard Practices for Method Validation in Forensic Toxicology as a

guideline (33). Briefly, the linear range for RA was 0.5-500 ng/mL. For MPH and EPH, the linear range was 0.5-200 ng/mL. The LOD was 0.5 ng/mL for RA and 0.25 ng/mL for MPH and EPH. Bias ranged from -12.7% to -4.8% for all analytes. Between-run precision was 3.6% to 6.9%CV for all analytes while maximum within-run precision was 4.7% to 12.5%CV (34).

Stability

The mean of the concentrations from the initial time point (T_0) was used as a baseline for each stability study. At each time point, the samples were extracted and analyzed following the procedure mentioned above. From the established baseline, stability was assessed as a %difference from this value by dividing the calculated concentration at each time point by the baseline concentration. The baseline concentration is displayed at 100% on the graphs. All concentration losses and gains are displayed on the graph as a change from 100%. Stability was considered acceptable between ±20%. The results of this study are summarized in Table 5.2. Due to the results of the long-term stability, an additional short-term stability experiment was executed to assess potential MPH degradation to RA. Due to this, QCs were made with only MPH and analyzed on the same method. MPH, EPH and RA were all monitored for quantification. For this study, MPH stability was assessed as a %difference from the established baseline. The results of this study are summarized in Table 5.3.

Table 5.2. Summary of data for MPH, EPH and RA stability at the LQC (15 ng/mL) and HQC (150 ng/mL). Data are displayed as %difference from 100% with the corresponding timepoint at which the analyte was deemed unstable.

	Room temperature (~25°C)		Refrigerated (4°C)		Frozen (-20°C)		Elevated (35°C)	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
d-MPH	-18.1 (24h)	-20.1 (24h)	-50.5 (2wk)	-54.8 (2wk)	-16.7 (5mo)	-14.3 (5mo)	-66.1 (24h)	-64.1 (24h)
l-MPH	-20.6 (24h)	-41.5 (24h)	-50.9 (2wk)	-56.0 (2wk)	-16.4 (5mo)	-16.9 (5mo)	-70.7 (24h)	-68.7 (24h)
d-EPH	-22.3 (48h)	-21.0 (48h)	-41.4 (2wk)	-47.1 (2wk)	-13.6 (5mo)	-13.4 (5mo)	-26.7 (24h)	-27.2 (24h)
l-EPH	-28.8 (72h)	-35.4 (72h)	-34.8 (2wk)	-53.5 (2wk)	-18.5 (5mo)	-12.1 (5mo)	-47.5 (48h)	-46.8 (48h)
RA	+52.8 (24h)	+35.8 (24h)	+40.8 (1wk)	+36.6 (1wk)	+18.5 (5mo)	+17.7 (5mo)	+116.7 (24h)	+143.5 (24h)

Table 5.3. Summary of data for MPH only at the LQC (15 ng/mL) and HQC (150 ng/mL). For MPH, data are displayed as %difference from 100% with the corresponding timepoint at which the analyte was deemed unstable. For RA, data are displayed as a %difference from 0% due to no RA being present in the T_0 samples. The timepoint at which RA became quantifiable is indicated.

	Room temperature (~25°C)		Refrigerated (4°C)		Frozen (-20°C)		Elevated (35°C)	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
d-MPH	-25.9 (48h)	-30.4 (48h)	-26.9 (2wk)	-28.7 (1wk)	+6.5 (1mo)	+1.3 (1mo)	-53.1 (24h)	-61.1 (24h)
l-MPH	-34.5 (48h)	-32.6 (48h)	-29.6 (1wk)	-29.6 (1wk)	+2. (1mo)	+0.8 (1mo)	-60.3 (24h)	-67.4 (24h)
RA	+7.2 (1wk)	+15 (24h)	-	+6.2 (48h)	-	-	+7.2 (1wk)	+15 (24h)

Results

Long term stability

Stability at room temperature

Graphs displaying analyte stability when stored at room temperature are found in Figure 5.1A and Figure 5.2A. At low and high concentrations, *d*- and *l*-MPH demonstrated instability after 24 hours with 18.1 and 20.6% loss from T_0, respectively, in the LQC and 20.1 and 41.5% loss from T_0, respectively, in the HQC. The *d*- and *l*-EPH enantiomers were stable for 48 and 72h, respectively, with 22.3 and 28.8% loss from T_0 in the LQC and 21 and 35.4% loss from T_0 in the HQC. RA concentrations increased (from T_0) as much as 53% after 24h and 205% by 5 months (indicated on the graphs as 153 and 305%, respectively).

Stability at refrigerated temperature

Graphs displaying analyte stability when stored at refrigerated temperature are found in Figure 5.1B and Figure 5.2B. At low and high concentrations, *d*- and *l*-MPH demonstrated instability after two weeks with 50.5 and 50.9% loss, respectively, in the LQC and 54.8 and 56% loss, respectively, in the HQC. The *d*- and *l*-EPH enantiomers also demonstrated instability after two weeks with 41.4 and 34.8% loss in the LQC and 47.1 and 43.5% loss in the HQC, respectively. RA demonstrated stability for at least 72 hours with a 17 and 16% gain in the LQC and HQC, respectively. RA concentrations increased 40.8 and 36.6% in the LQC and HQC, respectively, after one week and increased as much as 79.4% by 5 months.

Stability at frozen temperature

Graphs displaying analyte stability when stored at frozen temperature are found in Figure 5.1C and Figure 5.2C. At low and high concentrations, *d*- and *l*-MPH demonstrated stability for 5 months with only a maximum loss of 16.7 and 16.4% loss, respectively, in the LQC and 14.3 and 16.9% loss, respectively, in the HQC. The *d*- and *l*-EPH enantiomers were also stable for 5 months with a maximum of 13.6 and 18.5% loss in the LQC and 13.4% and 12.1% loss in the HQC, respectively. RA concentrations remained stable for 5 months with a maximum gain of 18.5% and 17.7% in the LQC and HQC, respectively.

Stability at elevated temperature

Graphs displaying analyte stability when stored at elevated temperature are found in Figure 5.1D and Figure 5.2D. At low and high concentrations, *d*- and *l*-MPH demonstrated instability after 24 hours with 66.1 and 70.7% loss, respectively, in the LQC and 64.1 and 68.7% loss, respectively, in the HQC. The *d*- and *l*-EPH enantiomers demonstrated instability after 24 and 48h, respectively, with 26.7 and 47.5% loss in the LQC and 27.2 and 46.8% loss in the HQC. RA concentrations increased as much as 1143% after 24h and 242% by 5 months.

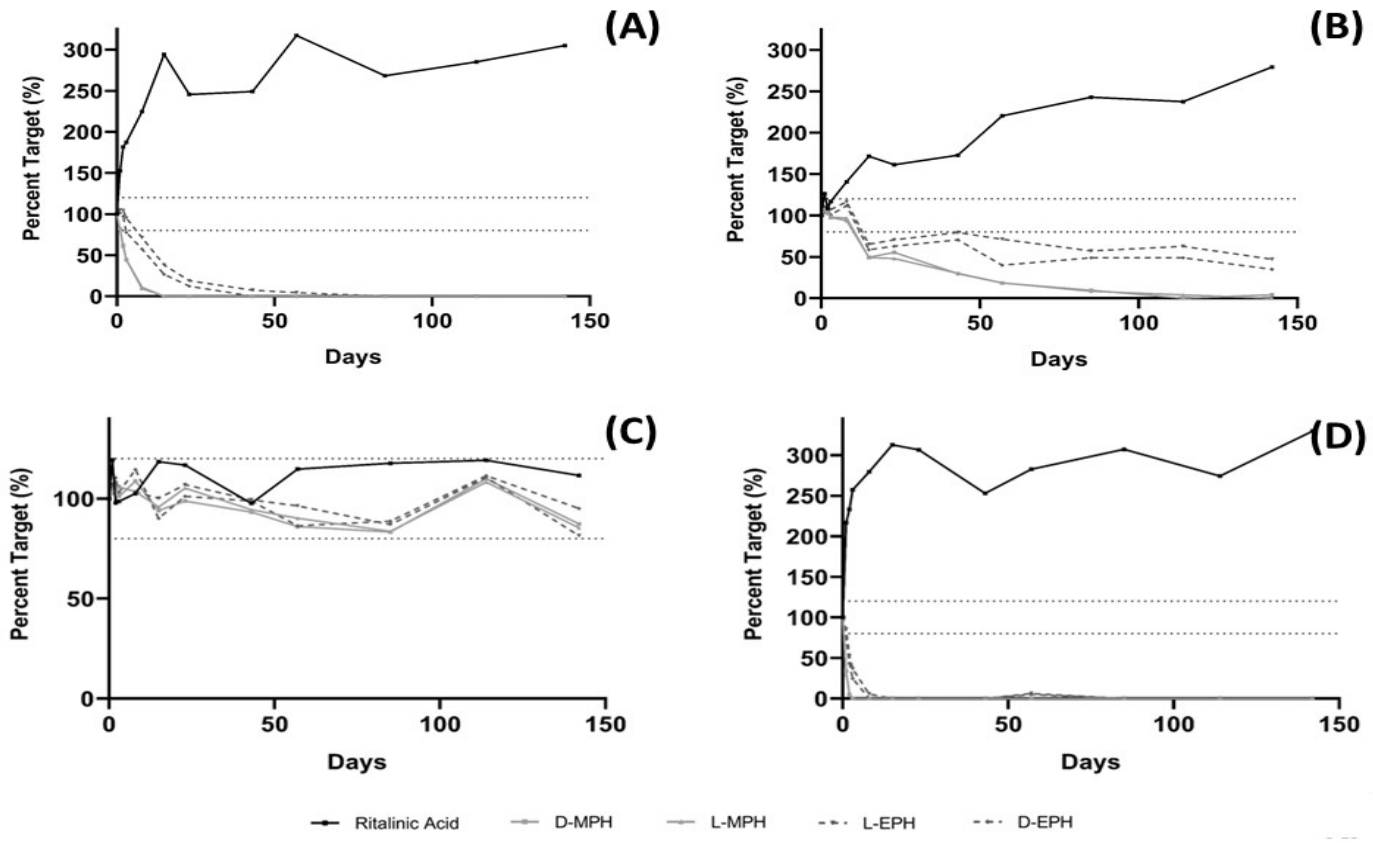

Figure 5.1. MPH, EPH and RA stability at the LQC (15 ng/mL) at room temperature (~25°C) (A), refrigerated temperature (4°C) (B), frozen temperature (-20°C) (C) and elevated temperature (35°C) (D)

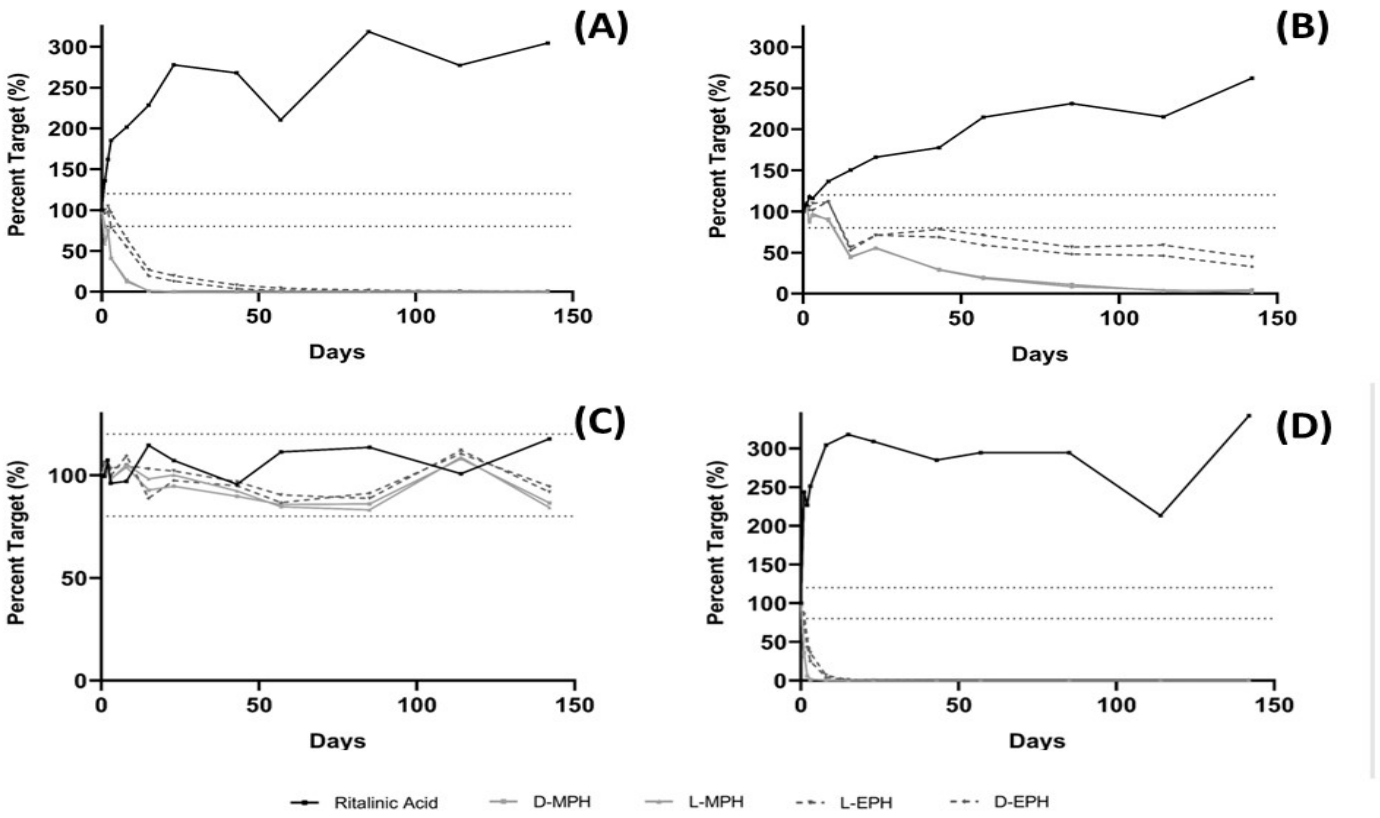

Figure 5.2. MPH, EPH and RA stability at the HQC (150 ng/mL) at room temperature (~25°C) (A), refrigerated temperature (4°C) (B), frozen temperature (-20°C) (C) and elevated temperature (35°C) (D)

MPH short term stability

Stability at room temperature

Graphs displaying analyte concentrations over time when stored at room temperature are found in Figure 5.3A and Figure 5.4A. At low and high concentrations, *d*- and *l*-MPH demonstrated instability after 48 hours with 25.9 and 34.5% loss, respectively, in the LQC and 30.4 and 32.6% loss, respectively, in the HQC. In the LQC containing 15 ng/mL MPH only, RA was present at 7.2 ng/mL after one week and rose to 9.5 ng/mL by one month. The enantiomers of MPH fully degraded within two weeks. In the HQC containing 150 ng/mL MPH only, RA was quantified at 15 ng/mL after 24 hours and increased to 126 ng/mL by one month. By one month, *d*-MPH was 2.2 ng/mL while *l*-MPH had fully degraded after 3 weeks.

Stability at refrigerated temperature

Graphs displaying analyte concentrations over time when stored at refrigerated temperature are found in Figure 5.3B and Figure 5.4B. At low concentrations, *d*- and *l*-MPH demonstrated instability after two weeks and one week with 26.9 and 21.3% loss, respectively. At the high concentrations, *d*- and *l*-MPH demonstrated instability after one week 28.7 and 29.6% loss, respectively, in the HQC. In the LQC, RA never rose into quantifiable range. In the HQC, RA was present at 6.2 ng/mL within 48 hours and increased to 70.1 ng/mL by one month. The enantiomers of MPH remained in quantifiable range for one month in both low and high concentrations.

Stability at frozen temperature

Graphs displaying analyte concentrations over time when stored at frozen temperature are found in Figure 5.3C and Figure 5.4C. At low and high concentrations, *d*- and *l*-MPH demonstrated stability for 1 month with ±15.1% difference from the target concentration. In the LQC, RA never rose into quantifiable range. In the HQC, RA was quantified at 5.4 ng/mL at one month. The enantiomers of MPH remained in quantifiable range for one month in both low and high concentrations.

Stability at elevated temperature

Graphs displaying analyte concentrations over time when stored at elevated temperature are found in Figure 5.3D and Figure 5.4D. At low concentrations and high concentrations, *d*- and *l*-MPH demonstrated instability after 24 hours with 53.1 and 60.3% loss, respectively, in the LQC and 61.1 and 67.4% loss, respectively, in the HQC. In the LQC, RA was present at 7.2 ng/mL after one week and rose to 9.5 ng/mL by one month. The enantiomers of MPH fully degraded within two weeks. In the HQC, RA was 15 ng/mL after 24 hours and increased to 126 ng/mL by one month. By one month, *d*-MPH was 2.2 ng/mL while *l*-MPH had fully degraded after 3 weeks.

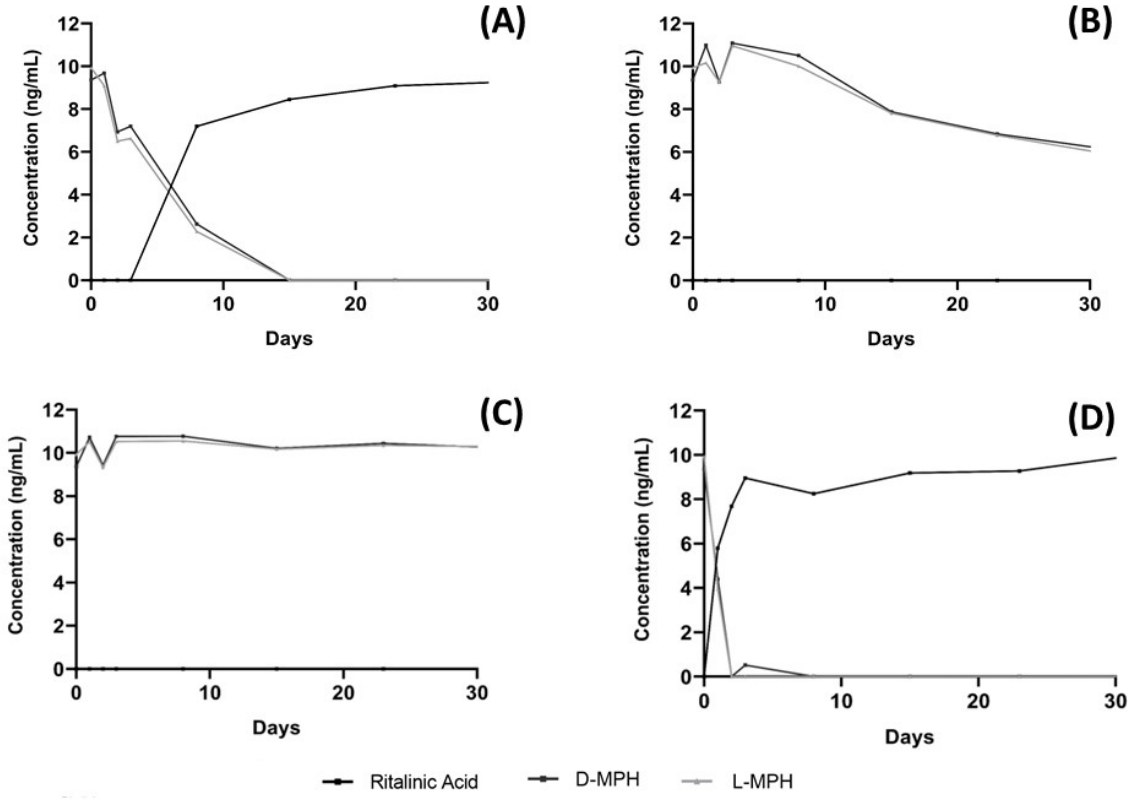

Figure 5.3. MPH and RA concentration vs time at the LQC (15 ng/mL MPH) at room temperature (~25°C) (A), refrigerated temperature (4°C) (B), frozen temperature (-20°C) (C) and elevated temperature (35°C) (D)

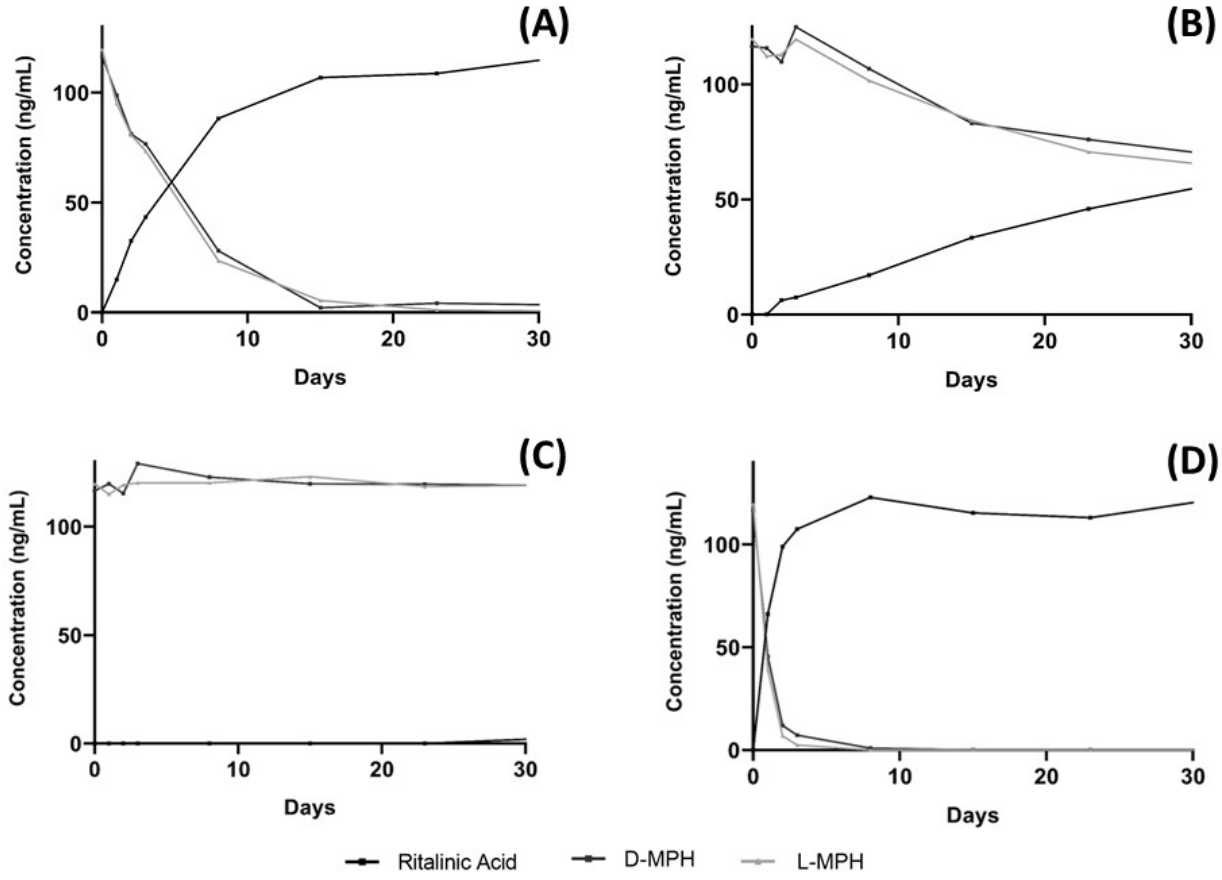

Figure 5.4. MPH and RA concentration vs time at the HQC (150 ng/mL MPH) at room temperature (~25°C) (A), refrigerated temperature (4°C) (B), frozen temperature (-20°C) (C) and elevated temperature (35°C) (D)

Discussion

When assessing stability, MPH and EPH degradation occurred within days at room temperature, within a week in refrigeration, and within one day for elevated temperatures. MPH, EPH, and RA were considered stable when blood was stored under frozen conditions. While RA also displayed instability, samples resulted in increasing RA concentrations over time.

In the frozen and refrigerated temperatures, both enantiomers of MPH and EPH demonstrated the same stability pattern. In frozen conditions, the enantiomers were stable for the entire 5 month study. In refrigerated temperatures, the enantiomers of both analytes remained stable for at least one week. When assessing elevated temperatures, only *l*-EPH remained stable for at least 24 hours. Additionally, *d* and *l*-EPH remained stable, at 48 hours and 72 hours, respectively, for a longer period at room temperature as compared to MPH (<24 hours). These data show that of the enantiomers, *l*-EPH is the most stable though all the enantiomers responded similarly throughout the study. As predicted in previous studies, this study demonstrates that EPH stability is similar to that of MPH (25, 32).

During experiments with all analytes, RA concentrations rose. Overall, the long-term and short-term trends for MPH were very similar. For room temperature, MPH and EPH both displayed instability and degradation within 48 h. These results are similar to those of Josefsson et al. that found MPH to be unstable at room temperature (31). Additionally, MPH was only stable in blood or plasma at ambient temperature for 4h or 6h in other studies (6, 15, 28). Zhu et al. found EPH to be stable for 4 h at ambient temperature (6). RA also displayed instability with increasing concentrations over this

time as observed with Josefsson et al. (31). Though there was minor variability when analyzing refrigerated temperatures, the enantiomers of MPH and EPH only remained stable for one week in our study. Zhu et al demonstrated MPH and EPH stability at 4°C for 24 h (6). In our short-term study, *d*-MPH remained stable for two weeks. When assessing frozen temperatures, all analytes remained stable throughout our study. The results of this study agree with Thomsen et al., Lin et al., and Joseffson et al. (15, 28, 31). Elevated temperatures resulted in degradation within 24 hours for all analytes in our study with the exception of *l*-EPH. Overall, the optimal storage condition for these analytes in blood is under frozen temperatures. This study shows that these analytes remain stable for at least 5 months in this condition and agrees with the results of Seçilir et al (30). As seen with the room temperature and refrigerated temperature, the RA concentrations increased in our study while MPH and EPH concentrations decreased. It was hypothesized that the concentration of RA increased due to breakdown of MPH to RA in the blood. The short-term stability study used samples that only contained MPH. During these experiments, RA was produced in MPH-fortified blood. In the LQC, the RA concentrations remained at <LOQ in both refrigerated and frozen temperatures. In the HQC, RA was detected and quantified within 48 h at 4°C. This value increased over the time of the study while the value of MPH decreased. At -20°C, RA was quantifiable within one month. At room temperature, RA was detectable within one week and 24 hours for the LQC and HQC, respectively. At elevated temperatures, RA became quantifiable within 24 hours and MPH fell to <LOQ within one week for LQC and 2 weeks for HQC. Overall, these data demonstrate that RA can be produced in samples containing MPH. When analyzing toxicological samples, RA concentrations may be

inaccurate as the concentrations could be a result of MPH breakdown and thus makes MPH concentration interpretation more difficult. This shows why keeping forensic samples in correct storage conditions can be vital to quantification of samples.

Conclusion

This study analyzed *d,l*-MPH, *d,l*-EPH and RA in blood over a 5 month period. The results found that frozen temperature is the optimal storage condition for these analytes. All analytes remained stable within this study. Additionally, a short-term stability study analyzing MPH alone displayed the metabolite, RA, in the fortified blood samples. This is important as quantitation values may be inaccurate in forensic samples due to breakdown products. This study shows the importance of stability studies and gives useful information on storage conditions for these analytes in blood.

References

1. Ritz, M.C., Lamb, R.J., Goldberg, S.R. and Kuhar, M.J. (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science, 237, 1219-23.
2. Ramos, L., Bakhtiar, R. and Tse, F.L.S. (2000) Liquid-liquid extraction using 96-well plate format in conjunction with liquid chromatography/tandem mass spectrometry for quantitative determination of methylphenidate (Ritalin®) in human plasma. Rapid Communications in Mass Spectrometry, 14, 740-745.
3. Patrick, K.S. and Markowitz, J.S. (1997) Pharmacology of methylphenidate, amphetamine enantiomers and pemoline in attention-deficit hyperactivity disorder. Human Psychopharmacology: Clinical and Experimental, 12, 527-546.
4. Wenthur, C.J. (2016) Classics in Chemical Neuroscience: Methylphenidate. ACS Chemical Neuroscience, 7, 1030-1040.
5. Wilens, T.E., Morrison, N.R. and Prince, J. (2011) An update on the pharmacotherapy of attention-deficit/hyperactivity disorder in adults. Expert Rev Neurother, 11, 1443-65.
6. Zhu, H.-J., Patrick, K.S. and Markowitz, J.S. (2011) Enantiospecific determination of dl-methylphenidate and dl-ethylphenidate in plasma by liquid chromatography–tandem mass spectrometry: Application to human ethanol interactions. Journal of Chromatography B, 879, 783-788.
7. Arnold, L.E. (2000) Methyiphenidate vs. amphetamine: Comparative review. Journal of Attention Disorders, 3, 200-211.

8. Wu, L.-T., Pilowsky, D.J., Schlenger, W.E. and Galvin, D.M. (2007) Misuse of methamphetamine and prescription stimulants among youths and young adults in the community. Drug and alcohol dependence, 89, 195-205.

9. Ding, Y.S., Fowler, J.S., Volkow, N.D., Dewey, S.L., Wang, G.J., Logan, J., et al. (1997) Chiral drugs: comparison of the pharmacokinetics of [11C]d-threo and l-threo-methylphenidate in the human and baboon brain. Psychopharmacology, 131, 71-78.

10. Ding, Y.-S., Gatley, S.J., Thanos, P.K., Shea, C., Garza, V., Xu, Y., et al. (2004) Brain kinetics of methylphenidate (Ritalin) enantiomers after oral administration. Synapse, 53, 168-175.

11. Patrick, K.S., Caldwell, R.W., Ferris, R.M. and Breese, G.R. (1987) Pharmacology of the enantiomers of threo-methylphenidate. Journal of Pharmacology and Experimental Therapeutics, 241, 152.

12. Ramos, L., Bakhtiar, R., Majumdar, T., Hayes, M. and Tse, F. (1999) Liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry enantiomeric separation of dl-threo-methylphenidate, (Ritalin®) using a macrocyclic antibiotic as the chiral selector. Rapid Communications in Mass Spectrometry, 13, 2054-2062.

13. Markowitz, J.S. and Patrick, K.S. (2008) Differential pharmacokinetics and pharmacodynamics of methylphenidate enantiomers: does chirality matter? J Clin Psychopharmacol, 28, S54-61.

14. Bakhtiar, R., Ramos, L. and Tse Francis, L.S. (2003) Toxicokinetic assessment of methylphenidate (Ritalin®) in a 13-week oral toxicity study in dogs. Biomedical Chromatography, 18, 45-50.

15. Thomsen, R., Rasmussen, H.B., Linnet, K. and the, I.C. (2012) Enantioselective Determination of Methylphenidate and Ritalinic Acid in Whole Blood from Forensic Cases Using Automated Solid-Phase Extraction and Liquid Chromatography–Tandem Mass Spectrometry. Journal of Analytical Toxicology, 36, 560-568.

16. Paterson, S.M., Moore, G.A., Florkowski, C.M. and George, P.M. (2012) Determination of methylphenidate and its metabolite ritalinic acid in urine by liquid chromatography/tandem mass spectrometry. Journal of Chromatography B, 881-882, 20-26.

17. Srinivas, N.R., Hubbard, J.W., McKay, G., Hawes, E.M. and Midha, K.K. (1991) In vitro hydrolysis of RR, SS-threo-methylphenidate by blood esterases—differential and enantioselective interspecies variability. Chirality, 3, 99-103.

18. Srinivas, N.R., Hubbard, J.W., Korchinski, E.D. and Midha, K.K. (1992) Stereoselective Urinary Pharmacokinetics of dl-threo-Methylphenidate and Its Major Metabolite in Humans. Journal of Pharmaceutical Sciences, 81, 747-749.

19. Patrick, K.S., Straughn, A.B., Minhinnett, R.R., Yeatts, S.D., Herrin, A.E., DeVane, C.L., et al. (2007) Influence of Ethanol and Gender on Methylphenidate Pharmacokinetics and Pharmacodynamics. Clinical pharmacology and therapeutics, 81, 346-353.

20. Iden, C.R. and Hungund, B.L. (1979) A chemical ionization selected ion monitoring assay for methylphenidate and ritalinic acid. Biomed Mass Spectrom, 6, 422-6.

21. Chan, Y.M., Soldin, S.J., Swanson, J.M., Deber, C.M., Thiessen, J.J. and MacLeod, S. (1980) Gas chromatographic/mass spectrometric analysis of methylphenidate (Ritalin) in serum. Clinical Biochemistry, 13, 266-272.

22. Portoghese, P.S. and Malspeis, L. (1961) Relative Hydrolytic Rates of Certain Alkyl (b) dl-α-(2-Piperidyl)-phenylacetates. Journal of Pharmaceutical Sciences, 50, 494-501.

23. Patrick, K.S., Corbin, T.R. and Murphy, C.E. (2014) Ethylphenidate as a Selective Dopaminergic Agonist and Methylphenidate–Ethanol Transesterification Biomarker. Journal of Pharmaceutical Sciences, 103, 3834-3842.

24. Parekh, P.K., Ozburn, A.R. and McClung, C.A. (2015) Circadian clock genes: Effects on dopamine, reward and addiction. Alcohol, 49, 341-349.

25. Parks, C., McKeown, D. and Torrance, H.J. (2015) A review of ethylphenidate in deaths in east and west Scotland. Forensic Science International, 257, 203-208.

26. Bailey, G.P., Ho, J.H., Hudson, S., Dines, A., Archer, J.R., Dargan, P.I., et al. (2015) Nopaine no gain: Recreational ethylphenidate toxicity. Clinical Toxicology, 53, 498-499.

27. Epstein, T., Patsopoulos, N.A. and Weiser, M. (2014) Immediate-release methylphenidate for attention deficit hyperactivity disorder (ADHD) in adults. Cochrane Database Syst Rev. 10.1002/14651858.CD005041.pub2, Cd005041.

28. N Lin, S., M. Andrenyak, D., Moody, D. and L. Foltz, R. (1999) Enantioselective Gas Chromatography-Negative Ion Chemical Ionization Mass Spectrometry for Methylphenidate in Human Plasma.

29. Wargin, W., Patrick, K., Kilts, C., Gualtieri, C.T., Ellington, K., Mueller, R.A., et al. (1983) Pharmacokinetics of methylphenidate in man, rat and monkey. Journal of Pharmacology and Experimental Therapeutics, 226, 382-386.

30. Seçilir, A., Schrier, L., Bijleveld, Y.A., Toersche, J.H., Jorjani, S., Burggraaf, J., et al. (2013) Determination of methylphenidate in plasma and saliva by liquid chromatography/tandem mass spectrometry. Journal of Chromatography B, 923-924, 22-28.

31. Josefsson, M. and Rydberg, I. (2011) Determination of methylphenidate and ritalinic acid in blood, plasma and oral fluid from adolescents and adults using protein precipitation and liquid chromatography tandem mass spectrometry—A method applied on clinical and forensic investigations. Journal of Pharmaceutical and Biomedical Analysis, 55, 1050-1059.

32. Krueger, J., Sachs, H., Musshoff, F., Dame, T., Schaeper, J., Schwerer, M., et al. (2014) First detection of ethylphenidate in human fatalities after ethylphenidate intake. Forensic Science International, 243, 126-129.

33. ASB/ANSI. (2019) ANSI/ASB Standard 036, First Edition. In Standard Practices for Method Validation in Forensic Toxicology.

34. Smith, C.R., Swortwood, Madeleine J. (2021) Chiral Separation and Analysis of Methylphenidate, Ethylphenidate and Ritalinic Acid in Blood by Liquid

Chromatography/Mass Spectrometry (LC/MS/MS). In American Academy of Forensic Sciences, Houston, TX* (virtual).

CHAPTER VI

CONCLUSIONS

With the rise of cognitive stimulant abuse along with the emergence of NPS, there are problems at hand when it comes to analysis of these drugs. Toxicologists must keep up with these emerging trends as the drug community and drug market are constantly evolving and users continue to seek new mechanisms for achieving euphoria, increasing focus, and beating a drug test. To overcome these issues, analytical methods must be developed to detect and quantify drugs of abuse in various biological matrices. Additionally, isomeric and enantiomeric drugs such as methylphenidate (MPH) may have varying effects on the body and must be enantiomerically separated to better understand their pharmacodynamic and pharmacokinetic (PD/PK) properties. The current study offers valuable information on chiral separation of MPH, ethylphenidate (EPH), as well as ritalinic acid (RA), using a chiral column, solid-phase extraction (SPE) and liquid chromatography-mass spectrometry (LC-MS/MS). It offers an alternative chromatographic technique, supercritical fluid-mass spectrometry (SFC-MS/MS), as a different approach to chiral separation and analysis. Chiral columns are more effective, cost efficient, and require less hazardous chemicals compared to derivatizing agents. Similarly, SPE requires less solvent consumption and is more selective than traditional liquid-liquid extraction. This current study also presents an analytical technique to detect common ADHD medications in oral fluid (OF). OF is a rapid, non-invasive matrix that allows for on-site collection. OF may indicate recent drug use and has applicability to driving under the influence of drugs (DUID) cases due to its simple collection. This on-site collection can be used in both clinical and forensic settings to detect use of these

cognitive stimulants, especially on college campuses where studies indicate rising abuse. The current study provides a method that allows for detection of cognitive stimulants in oral fluid and applicability is shown by analysis of authentic samples from college students currently prescribed these medications. Lastly, stability studies are important to understand accurate quantitative values when assessing toxicological findings, especially with emerging NPS. It is important to know if drugs are unstable or if absence of a finding may indicate degradation. The current study conducted a five-month stability study on these cognitive stimulants and found that they were only stable at frozen temperatures (-20ºC) for this period. At elevated and ambient temperatures, MPH degraded to ritalinic acid and if not properly handled or stored, inaccurate quantitative values may be obtained. With the use of the analytical methods described above, the clinical and forensic toxicology communities can develop viable approaches to properly detect and analyze cognitive stimulants in various biological matrices, including blood and oral fluid. Additionally, as EPH is emerging as an NPS, the techniques in this study can provide valuable information to the forensic community about this drug and others alike. This study aimed to address analytical gaps in the literature by developing enantioselective separation techniques for analysis of MPH and its metabolites, EPH and RA, in various biological samples. This study provided validated analytical methods to detect and quantify MPH and other cognitive stimulants using LC-MS/MS for blood and oral fluid and SFC-MS/MS for blood. Lastly, this study analyzed MPH instability to better understand degradation to address proper storage and handling of forensic samples to ensure accurate quantification for data interpretation. As EPH is recognized as biomarker for MPH as well as a NPS, additional research needs to be done to understand

the PD/PK of this analyte and its enantiomers and the effect it has on the body. Additionally, the current study can be used to better understand potential abuse of cognitive stimulants on the college campus. Due to ease of collection of oral fluid, this method can be used to quantify common medications that may be used illicitly on college campuses.

REFERENCES

2011. EMCDDA. Europol 2011 Annual Report on the implementation of Council Decision. https://www.europol.europa.eu/sites/default/files/publications/emcddaeuropol_annual_report_2011_2012_final.pdf. (Accessed April 2021)

2012. Adderall (dextroamphetamine saccharate, amphetamine aspartate, dextroamphetamine sulfate and amphetamine sulfate tablet) [product information]. . In: Pharmaceuticals, T., (Ed), North Wales, PA.

2013. EMCDDA. 2013 Europol 2013 Annual Report on the implementation of Council Decision. http://www.emcdda.europa.eu/attachements.cfm/att_229598_EN_TDAN14001ENN.pdf. (Accessed April 2021)

2015. Home Office. The Misuse of Drugs Act 1971 (Temporary Class Drug) Order 2015. http://www.legislation.gov.uk/uksi/2015/1027/pdfs/uksi_20151027_en.pdf. (Accessed April 202)

2019. Common ADHD Medications & Treatment for Children. American Academy of Pediatrics.

Adamowicz, P., Tokarczyk, B., 2019. Screening Analysis for Designer Stimulants by LC-MS/MS. In: Langman, L. J., Snozek, C. L. H., (Eds), LC-MS in Drug Analysis: Methods and Protocols. Springer New York, New York, NY, pp. 165-180.

Adler, L.A., Alperin, S., Leon, T. and Faraone, S.V. 2016. Pharmacokinetic and Pharmacodynamic Properties of Lisdexamfetamine in Adults with Attention-Deficit/Hyperactivity Disorder. Journal of Child and Adolescent Psychopharmacology, 27, 196-199.

Advokat, C., 2007. Literature review: Update on amphetamine neurotoxicity and its relevance to the treatment of ADHD. Journal of Attention Disorders 11, 8-16.

Advisory Committee on the Misuse of Drugs, 2011. Considerations of the novel psychoactive subsances ('Legal Highs'). https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/119139/acmdnps2011.pd

Advokat, C., 2009. What exactly are the benefits of stimulants for ADHD? Journal of Attention Disorders 12, 495-498.

Advokat, C., 2010. What are the cognitive effects of stimulant medications? Emphasis on adults with attention-deficit/hyperactivity disorder (ADHD). Neuroscience & Biobehavioral Reviews 34, 1256-1266.

Alothman, Z.A., Alanazi, A.G., Suhail, M. and Ali, I. 2020. HPLC enantio-separation and chiral recognition mechanism of quinolones on vancomycin CSP. Journal of Chromatography B, 1157, 122335

Antshel, K. M., Hargrave, T. M., Simonescu, M., Kaul, P., Hendricks, K., Faraone, S. V., 2011. Advances in understanding and treating ADHD. BMC Medicine 9, 72.

Arnold, L.E. 2000. Methylphenidate vs. amphetamine: Comparative review. Journal of Attention Disorders, 3, 200-211.

Arnsten, A. F., 2007. Catecholamine and second messenger influences on prefrontal cortical networks of "representational knowledge": a rational bridge between genetics and the symptoms of mental illness. Cerebral Cortex, 17, i6-i15.

Arnsten, A. F., Paspalas, C. D., Gamo, N. J., Yang, Y., Wang, M., 2010. Dynamic network connectivity: a new form of neuroplasticity. Trends in Cognitive Sciences, 14, 365-375.

Arnsten, A. F., Pliszka, S. R., 2011. Catecholamine influences on prefrontal cortical function: relevance to treatment of attention deficit/hyperactivity disorder and related disorders. Pharmacology Biochemistry and Behavior, 99, 211-216.

Arvidsson, M., Dahl, M.-L., Beck, O., Ackehed, G., Nordin, K., Rosenborg, S., 2020. Pharmacokinetics of methylphenidate and ritalinic acid in plasma correlations with exhaled breath and oral fluid in healthy volunteers. European Journal of Clinical Pharmacology, 76, 229-237.

ASB/ANSI. 2019. ANSI/ASB Standard 036, First Edition. In Standard Practices for Method Validation in Forensic Toxicology.

Aoyama, T., Kotaki, H., Honda, Y. and Nakagawa, F. 1990. Kinetic analysis of enantiomers of threo-methylphenidate and its metabolite in two healthy subjects after oral administration as determined by a gas chromatographic-mass spectrometric method. Journal of Pharmaceutical Sciences, 79, 465-469.

Babcock, Q., Byrne, T., 2000. Student perceptions of methylphenidate abuse at a public liberal arts college. Journal of American College Health, 49, 143-145.

Bailey, G. P., Ho, J. H., Hudson, S., Dines, A., Archer, J. R., Dargan, P. I., Wood, D. M., 2015. Nopaine no gain: Recreational ethylphenidate toxicity. Clinical Toxicology 53, 498-499.

Bakhtiar, R., Ramos, L., Tse, F. L. S., 2002. Quantification of methylphenidate in rat, rabbit and dog plasma using a chiral liquid-chromatography/tandem mass spectrometry method: Application to toxicokinetic studies. Analytica Chimica Acta 469, 261-272.

Bakhtiar, R., Ramos, L. and Tse Francis, L.S. 2003. Toxicokinetic assessment of methylphenidate (Ritalin®) in a 13-week oral toxicity study in dogs. Biomedical Chromatography, 18, 45-50.

Barceló, B., Gomila, I., Rotolo, M., Marchei, E., Kyriakou, C., Pichini, S., Roset, C., Elorza, M., Busardò, F., 2017. Intoxication caused by new psychostimulants: analytical methods to disclose acute and chronic use of benzofurans and ethylphenidate. International Journal of Legal Medicine 131, 1543.

Barkley, R. A., Cunningham, C. E., 1978. Do stimulant drugs improve the academic performance of hyperkinetic children? A review of outcome studies. Clinical Pediatrics 17, 85-92.

Barrett, S. P., Pihl, R. O., 2002. Oral methylphenidate-alcohol co-abuse. Journal of Clinical Psychopharmacology, 22, 633-634.

Bartl, J., Palazzesi, F., Parrinello, M., Hommers, L., Riederer, P., Walitza, S., Grünblatt, E., 2017. The impact of methylphenidate and its enantiomers on dopamine synthesis and metabolism in vitro. Progress in Neuro-Psychopharmacology and Biological Psychiatry 79, 281-288.

Beck, O., Stephanson, N., Sandqvist, S., Franck, J., 2014. Determination of Amphetamine and Methylphenidate in Exhaled Breath of Patients Undergoing Attention-Deficit/Hyperactivity Disorder Treatment. Therapeutic Drug Monitoring, 36.

Bell, G., Griffin Iii, W., Patrick, K., 2011. Transdermal and oral dl-methylphenidate – ethanol interactions in C57BL/6J mice: Locomotor activity and brain d-, l-methylphenidate and l-ethylphenidate concentrations. The FASEB Journal 25, lb427-lb427.

Bentley, J., Snyder, F., Brown, S. D., Brown, R., Pond, B. B., 2015. Sex differences in the kinetic profiles of d-and l-methylphenidate in the brains of adult rats. European Review for Medical and Pharmacological Sciences, 19, 2514-2519.

Berger, I., 2011. Diagnosis of attention deficit hyperactivity disorder: much ado about something. Israel Medical Association Journal, 13, 571-574.

Berridge, C. W., Devilbiss, D. M., Andrzejewski, M. E., Arnsten, A. F., Kelley, A. E., Schmeichel, B., Hamilton, C., Spencer, R. C., 2006. Methylphenidate preferentially increases catecholamine neurotransmission within the prefrontal cortex at low doses that enhance cognitive function. Biological Psychiatry, 60, 1111-1120.

Biederman, J., 2003. Pharmacotherapy for attention-deficit/hyperactivity disorder (ADHD) decreases the risk for substance abuse: findings from a longitudinal follow-up of youths with and without ADHD. Journal of Clinical Psychiatry, 64, 3-8.

Biederman, J., Boellner, S. W., Childress, A., Lopez, F. A., Krishnan, S., Zhang, Y., 2007. Lisdexamfetamine dimesylate and mixed amphetamine salts extended-release in children with ADHD: a double-blind, placebo-controlled, crossover analog classroom study. Biological Psychiatry, 62, 970-976.

Biederman, J., Faraone, S. V., Spencer, T. J., Mick, E., Monuteaux, M. C., Aleardi, M., 2006. Functional impairments in adults with self-reports of diagnosed ADHD: A controlled study of 1001 adults in the community. Journal of Clinical Psychiatry, 67, 524-540.

Biederman, J., Monuteaux, M. C., Spencer, T., Wilens, T. E., Faraone, S. V., 2009. Do stimulants protect against psychiatric disorders in youth with ADHD? A 10-year follow-up study. Pediatrics, 124, 71-78.

Biederman, J., Spencer, T., 2002. Methylphenidate in treatment of adults with Attention-Deficit/Hyperactivity Disorder. Journal of Attention Disorders, 6, S101-107

Biederman, J., Wilens, T., Mick, E., Spencer, T., Faraone, S. V., 1999. Pharmacotherapy of attention-deficit/hyperactivity disorder reduces risk for substance use disorder. Pediatrics, 104, e20-e20

Bluelight. Ethylphenidate - anyone tried it yet? http://www.bluelight.org/vb/archive/index.php/t-520608.html (Accessed April 2021)

Bluelight. 2013. The Ethylphenidate (Ethyl phenyl(piperidin-2-yl)acetate) Megathread V3. https://www.bluelight.org/xf/threads/the-ethylphenidate-ethyl-phenyl-piperidin-2-yl-acetate-megathread-v3.687516/ (Accessed April 2021)

Boellner, S.W., Stark, J.G., Krishnan, S. and Zhang, Y. 2010. Pharmacokinetics of lisdexamfetamine dimesylate and its active metabolite, d-amphetamine, with increasing oral doses of lisdexamfetamine dimesylate in children with attention-deficit/hyperactivity disorder: a single-dose, randomized, open-label, crossover study. Clinical Therapeutics, 32, 252-64

Bolea-Alamanac, B., Nutt, D., Adamou, M., Asherson, P., Bazire, S., Coghill, D., Heal, D., Müller-Sedgwick, U., Nash, J., Santosh, P., Sayal, K., Sonuga-Barke, E., Young, S., 2014a. Evidence-based guidelines for the pharmacological management of attention deficit hyperactivity disorder: Update on recommendations from the British Association for Psychopharmacology. Journal of Psychopharmacology, 28.

Bolea-Alamanac, B. M., Green, A., Verma, G., Maxwell, P., Davies, S. J. C., 2014b. Methylphenidate use in pregnancy and lactation: a systematic review of evidence. British Journal of Clinical Pharmacology, 77, 96-101.

Bourland, J. A., Martin, D. K., Mayersohn, M., 1997. Carboxylesterase-mediated transesterification of meperidine (Demerol) and methylphenidate (Ritalin) in the presence of [2H6]ethanol: preliminary in vitro findings using a rat liver preparation. Journal of Pharmaceutical Sciences, 86, 1494-1496.

Böttcher, M., Kühne, D. and Beck, O. 2019. Compliance testing of patients in ADHD treatment with lisdexamphetamine (Elvanse®) using oral fluid as specimen. Clinical Mass Spectrometry, 14, 99-105.

Breda, V., Rohde, L. A., Menezes, A. M. B., Anselmi, L., Caye, A., Rovaris, D. L., Vitola, E. S., Bau, C. H. D., Grevet, E. H., 2021. The neurodevelopmental nature of attention-deficit hyperactivity disorder in adults. British Journal of Psychiatry, 218, 43-50.

Butcher, S., Liptrot, J., Aburthnott, G., 1991. Characterisation of methylphenidate and nomifensine induced dopamine release in rat striatum using in vivo brain microdialysis. Neuroscience Letters, 122, 245-248.

Cantrell, F. L., Ogera, P., Mallett, P., McIntyre, I. M., 2014. Fatal oral methylphenidate intoxication with postmortem concentrations. Journal of Forensic Sciences, 59, 847-849.

Cantwell, D. P., Satterfield, J. H., 1978. The prevalence of academic underachievement in hyperactive children. Journal of Pediatric Psychology, 3, 168-171.

Carlson, C. L., Bunner, M. R., 1993. Effects of methylphenidate on the academic performance of children with attention-deficit hyperactivity disorder and learning disabilities. School Psychology Review, 22, 184-198.

Casale, J.F. and Hays, P.A. 2011. Ethylphenidate: an analytical profile. Microgram Journal, 8, 58-61.

Chan, Y., Soldin, S., Swanson, J., Deber, C., Thiessen, J., Macleod, S., 1980. Gas chromatographic/mass spectrometric analysis of methylphenidate (Ritalin) in serum. Clinical Biochemistry, 13, 266-272.

Childress, A. C., Berry, S. A., 2012. Pharmacotherapy of attention-deficit hyperactivity disorder in adolescents. Drugs, 72, 309-325.

Childress, A. C., Komolova, M., Sallee, F. R., 2019. An update on the pharmacokinetic considerations in the treatment of ADHD with long-acting methylphenidate and amphetamine formulations. Expert Opin Drug Metabolism Toxicology, 15, 937-974.

Combs, C. C., Hankins, E. L., Copeland, C. L., Brown, S. D., Pond, B. B., 2013. Quantitative determination of d- and l-threo enantiomers of methylphenidate in brain tissue by liquid chromatography-mass spectrometry. John Wiley & Sons, Ltd, Great Britain, p. 1587.

Comiran, E., Barreto, F., Meneghini Leonardo, Z., Carlos, G., Fröehlich Pedro, E. and Limberger Renata, P. 2016. Method validation and determination of lisdexamfetamine and amphetamine in oral fluid, plasma and urine by LC–MS/MS. Biomedical Chromatography, 31, e3812.

Comiran, E., Carlos, G., Barreto, F., Pechanksy, F., Fröehlich, P.E. and Limberger, R.P. 2021, Lisdexamfetamine and amphetamine pharmacokinetics in oral fluid, plasma, and urine after controlled oral administration of lisdexamfetamine. Biopharmaceutics and Drug Disposition, 42, 3-11.

Comiran, E., Kessler, F.H., Fröehlich, P.E. and Limberger, R.P. 2016. Lisdexamfetamine: A pharmacokinetic review. European Journal of Pharmaceutical Sciences, 89, 172-9

Cone, E.J. and Huestis, M.A. 2007. Interpretation of oral fluid tests for drugs of abuse. Annals of the New York Academy of the Sciences, 1098, 51-103.

Connor, D. F., Glatt, S. J., Lopez, I. D., Jackson, D., Melloni Jr, R. H., 2002. Psychopharmacology and aggression. I: A meta-analysis of stimulant effects on overt/covert aggression–related behaviors in ADHD. Journal of the American Academy of Child & Adolescent Psychiatry 41, 253-261.

Cortese, S., 2012. The neurobiology and genetics of attention-deficit/hyperactivity disorder (ADHD): what every clinician should know. European Journal of Paediatric Neurology, 16, 422-433.

Cortese, S., 2020. Pharmacologic Treatment of Attention Deficit-Hyperactivity Disorder. New England Journal of Medicine, 383, 1050-1056.

Cortese, S., D'Acunto, G., Konofal, E., Masi, G., Vitiello, B., 2017. New Formulations of Methylphenidate for the Treatment of Attention-Deficit/Hyperactivity Disorder: Pharmacokinetics, Efficacy, and Tolerability. CNS Drugs, 31, 149-160.

Darredeau, C., Barrett Sean, P., Jardin, B., Pihl Robert, O., 2007. Patterns and predictors of medication compliance, diversion, and misuse in adult prescribed methylphenidate users. Human Psychopharmacology: Clinical and Experimental, 22, 529-536.

de Cássia Mariotti, K., Rübensam, G., Barreto, F., Bica, V. C., Meneghini, L. Z., Ortiz, R. S., Froehlich, P. E., Limberger, R. P., 2014. Simultaneous Determination of Fenproporex, Diethylpropione and Methylphenidate in Oral Fluid by LC-MS/MS. Chromatographia, 77, 83-90.

Demontis, D., Walters, R. K., Martin, J., Mattheisen, M., Als, T. D., Agerbo, E., Baldursson, G., Belliveau, R., Bybjerg-Grauholm, J., Bækvad-Hansen, M., Cerrato, F., Chambert, K., Churchhouse, C., Dumont, A., Eriksson, N., Gandal, M., Goldstein, J. I., Grasby, K. L., Grove, J., Gudmundsson, O. O., Hansen, C. S., Hauberg, M. E., Hollegaard, M. V., Howrigan, D. P., Huang, H., Maller, J. B., Martin, A. R., Martin, N. G., Moran, J., Pallesen, J., Palmer, D. S., Pedersen, C. B., Pedersen, M. G., Poterba, T., Poulsen, J. B., Ripke, S., Robinson, E. B., Satterstrom, F. K., Stefansson, H., Stevens, C., Turley, P., Walters, G. B., Won, H., Wright, M. J., Andreassen, O. A., Asherson, P., Burton, C. L., Boomsma, D. I., Cormand, B., Dalsgaard, S., Franke, B., Gelernter, J., Geschwind, D., Hakonarson, H., Haavik, J., Kranzler, H. R., Kuntsi, J., Langley, K., Lesch, K. P., Middeldorp, C., Reif, A., Rohde, L. A., Roussos, P., Schachar, R., Sklar, P., Sonuga-Barke, E. J. S., Sullivan, P. F., Thapar, A., Tung, J. Y., Waldman, I. D., Medland, S. E., Stefansson, K., Nordentoft, M., Hougaard, D. M., Werge, T., Mors, O., Mortensen, P. B., Daly, M. J., Faraone, S. V., Børglum, A. D., Neale, B. M., 2019. Discovery of the first genome-wide significant risk loci for attention deficit/hyperactivity disorder. National Genetics, 51, 63-75.

DeSantis, A.D., Webb, E.M. and Noar, S.M. 2008. Illicit Use of Prescription ADHD Medications on a College Campus: A Multimethodological Approach. Journal of American College Health, 57, 315-324.

Desfontaine, V., Capetti, F., Nicoli, R., Kuuranne, T., Veuthey, J.-L., Guillarme, D., 2018. Systematic evaluation of matrix effects in supercritical fluid chromatography versus liquid chromatography coupled to mass spectrometry for biological samples. Journal of Chromatography B, 1079, 51-61.

Desfontaine, V., Veuthey, J.-L., Guillarme, D., 2016. Evaluation of innovative stationary phase ligand chemistries and analytical conditions for the analysis of basic drugs by supercritical fluid chromatography. Journal of Chromatography A, 1438, 244-253

Desrosiers, N. A., Huestis, M. A., 2019. Oral Fluid Drug Testing: Analytical Approaches, Issues and Interpretation of Results. Journal of Analytical Toxicology, 43, 415-443.

Ding, Y. S., Fowler, J. S., Volkow, N. D., Dewey, S. L., Wang, G. J., Logan, J., Gatley, S. J., Pappas, N., 1997. Chiral drugs: comparison of the pharmacokinetics of [11C]d-threo and l-threo-methylphenidate in the human and baboon brain. Psychopharmacology, 131, 71-78.

Ding, Y.-S., Gatley, S.J., Thanos, P.K., Shea, C., Garza, V., Xu, Y., et al. 2004. Brain kinetics of methylphenidate (Ritalin) enantiomers after oral administration. Synapse, 53, 168-175.

Dinis-Oliveira, R. J., 2017. Metabolomics of Methylphenidate and Ethylphenidate: Implications in Pharmacological and Toxicological Effects. European Journal of Drug Metabolism and Pharmacokinetics, 42, 11-16

Dopheide, J. A., Pliszka, S. R., 2009. Attention-deficit-hyperactivity disorder: an update. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 29, 656-679.

Drugs-forum. 2011. Ethylphenidate experiences. https://drugs-forum.com/threads/ethylphenidate-experiences.156994/ (Accessed April 2021)

Eichhorst, J., Etter, M., Lepage, J., Lehotay, D. C., 2004. Urinary screening for methylphenidate (Ritalin) abuse: a comparison of liquid chromatography–tandem mass spectrometry, gas chromatography–mass spectrometry, and immunoassay methods. Clinical Biochemistry, 37, 175-183.

Ermer, J., Haffey, M.B., Richards, C., Lasseter, K., Roesch, B., Purkayastha, J., et al. 2013. An open-label investigation of the pharmacokinetic profiles of lisdexamfetamine dimesylate and venlafaxine extended-release, administered alone and in combination, in healthy adults. Clinical Drug Investigation, 33, 243-254

Ermer, J.C., Haffey, M.B., Doll, W.J., Martin, P., Sandefer, E.P., Dennis, K., et al. 2012. Pharmacokinetics of lisdexamfetamine dimesylate after targeted gastrointestinal release or oral administration in healthy adults. Drug Metabolism and Disposition, 40, 290-297.

Epstein, T., Patsopoulos, N. A., Weiser, M., 2014. Immediate-release methylphenidate for attention deficit hyperactivity disorder (ADHD) in adults. Cochrane Database Systematic Review, Cd005041.

Erowid, Erowid experience vaults: ethylphenidate reports. https://www.erowid.org/experiences/subs/exp_Ethylphenidate.shtml.

Faraone, S. V., 2009. Using meta-analysis to compare the efficacy of medications for attention-deficit/hyperactivity disorder in youths. Pharmacy and Therapeutics, 34, 678.

Faraone, S. V., 2018. The pharmacology of amphetamine and methylphenidate: Relevance to the neurobiology of attention-deficit/hyperactivity disorder and other psychiatric comorbidities. Neuroscience and Biobehavioral Reviews, 87, 255-270.

Faraone, S. V., Banaschewski, T., Coghill, D., Zheng, Y., Biederman, J., Bellgrove, M. A., Newcorn, J. H., Gignac, M., Al Saud, N. M., Manor, I., Rohde, L. A., Yang, L., Cortese, S., Almagor, D., Stein, M. A., Albatti, T. H., Aljoudi, H. F., Alqahtani, M. M. J., Asherson, P., Atwoli, L., Bölte, S., Buitelaar, J. K., Crunelle, C. L., Daley, D., Dalsgaard, S., Döpfner, M., Espinet, S., Fitzgerald, M., Franke, B., Gerlach, M., Haavik, J., Hartman, C. A., Hartung, C. M., Hinshaw, S. P., Hoekstra, P. J., Hollis, C., Kollins, S. H., Sandra Kooij, J. J., Kuntsi, J., Larsson, H., Li, T., Liu, J., Merzon, E., Mattingly, G., Mattos, P., McCarthy, S., Mikami, A. Y., Molina, B. S. G., Nigg, J. T., Purper-Ouakil, D., Omigbodun, O. O., Polanczyk, G. V., Pollak, Y., Poulton, A. S., Rajkumar, R. P., Reding, A., Reif, A., Rubia, K., Rucklidge, J., Romanos, M., Ramos-Quiroga, J. A., Schellekens, A., Scheres, A., Schoeman, R., Schweitzer, J. B., Shah, H., Solanto, M. V., Sonuga-Barke, E., Soutullo, C., Steinhausen, H.-C., Swanson, J. M., Thapar, A., Tripp, G., van de Glind, G., van den Brink, W., Van der Oord, S., Venter, A., Vitiello, B., Walitza, S., Wang, Y., 2021. The World Federation of ADHD International Consensus Statement: 208 Evidence-based conclusions about the disorder. Neuroscience & Biobehavioral Reviews, 128, 789-818.

Faraone, S. V., Biederman, J., Monuteaux, M., Spencer, T., 2005. Long-term effects of extended-release mixed amphetamine salts treatment of attention-deficit/hyperactivity disorder on growth. Journal of Child & Adolescent Psychopharmacology, 15, 191-202.

Faraone, S. V., Buitelaar, J., 2010. Comparing the efficacy of stimulants for ADHD in children and adolescents using meta-analysis. European Child & Adolescent Psychiatry, 19, 353-364.

Fayyad, J., Sampson, N. A., Hwang, I., Adamowski, T., Aguilar-Gaxiola, S., Al-Hamzawi, A., Andrade, L. H. S. G., Borges, G., de Girolamo, G., Florescu, S., Gureje, O., Haro, J. M., Hu, C., Karam, E. G., Lee, S., Navarro-Mateu, F., O'Neill, S., Pennell, B.-E., Piazza, M., Posada-Villa, J., ten Have, M., Torres, Y., Xavier, M., Zaslavsky, A. M., Kessler, R. C., Adamowski, T., Aguilar-Gaxiola, S., Al-Hamzawi, A., Al-Kaisy, M., Subaie, A. A., Alonso, J., Altwaijri, Y., Andrade, L. H., Atwoli, L., Auerbach, R. P., Axinn, W. G., Benjet, C., Borges, G., Bossarte, R. M., Bromet, E. J., Bruffaerts, R., Bunting, B., Caffo, E., de Almeida, J. M. C., Cardoso, G., Cia, A. H., Chardoul, S., Chatterji, S., Filho, A. C., Cuijpers, P., Degenhardt, L., de Girolamo, G., de Graaf, R., de Jonge, P., Demyttenaere, K., Ebert, D. D., Evans-Lacko, S., Fayyad, J., Fiestas, F., Florescu, S., Forresi, B., Galea, S., Germine, L., Gilman, S. E., Ghimire, D. J., Glantz, M. D., Gureje, O., Haro, J. M., He, Y., Hinkov, H., Hu, C.-y., Huang, Y., Karam, A. N., Karam, E. G., Kawakami, N., Kessler, R. C., Kiejna, A., Koenen, K. C., Kovess-Masfety, V., Lago, L., Lara, C., Lee, S., Lepine, J.-P., Levav, I., Levinson, D., Liu, Z., Martins, S. S., Matschinger, H., McGrath, J. J., McLaughlin, K. A., Medina-Mora, M. E., Mneimneh, Z., Moskalewicz, J., Murphy, S. D., Navarro-Mateu, F., Nock, M. K., O'Neill, S., Oakley-Browne, M., Hans Ormel, J., Pennell, B.-E., Piazza, M., Pinder-Amaker, S., Piotrowski, P., Posada-Villa, J., Ruscio, A. M., Scott, K. M., Shahly, V., Silove, D., Slade, T., Smoller, J. W., Stagnaro, J. C., Stein, D. J., Street, A. E., Tachimori, H., Taib, N., Have, M. t.,

Thornicroft, G., Torres, Y., Viana, M. C., Vilagut, G., Wells, E., Williams, D. R., Williams, M. A., Wojtyniak, B., Zaslavsky, A. M., on behalf of the, W. H. O. W. M. H. S. C., 2017. The descriptive epidemiology of DSM-IV Adult ADHD in the World Health Organization World Mental Health Surveys. ADHD Attention Deficit and Hyperactivity Disorders, 9, 47-65.

Food and Drug Administration, 2017b. Cotempla XR-ODT (methylphenidate extended-release orally disintegrating tablets). https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/205489s000lbl.pdf.

Folprechtová, D., Kalíková, K., Kadkhodaei, K., Reiterer, C., Armstrong, D. W., Tesařová, E., Schmid, M. G., 2021. Enantioseparation performance of superficially porous particle vancomycin-based chiral stationary phases in supercritical fluid chromatography and high performance liquid chromatography; applicability for psychoactive substances. Journal of Chromatography A, 1637, 461846

UK Legal Highs Forum. Ethylphenidate/ethylcaine super detailed report. http://www.legalhighsforum.com/showthread.php?17351-Ethylphenidate-Ethylcaine-SUPER-DETAILED-Report (Accessed April 2021)

Fusar-Poli, P., Rubia, K., Rossi, G., Sartori, G., Balottin, U., 2012. Striatal dopamine transporter alterations in ADHD: pathophysiology or adaptation to psychostimulants? A meta-analysis. American Journal of Psychiatry, 169, 264-272

Gadow, K. D., 1983. Effects of stimulant drugs on academic performance in hyperactive and learning disabled children. Journal of Learning Disabilities, 16, 290-299.

Gamo, N. J., Wang, M., Arnsten, A. F., 2010. Methylphenidate and atomoxetine enhance prefrontal function through α2-adrenergic and dopamine D1 receptors. Journal of the American Academy of Child & Adolescent Psychiatry, 49, 1011-1023.

Gandhi, A., Beekman, C., Parker, R., Fang, L., Babiskin, A., Matta, M. K., 2018. Novel and rapid LC–MS/MS method for quantitative analysis of methylphenidate in dried blood spots. Bioanalysis, 10, 839-850.

Gibbons, S., 2012. 'Legal Highs' – novel and emerging psychoactive drugs: a chemical overview for the toxicologist. Clinical Toxicology, 50, 15-24.

Giorgetti, A., Barone, R., Pelletti, G., Garagnani, M., Pascali, J., Haschimi, B., Auwärter, V., 2021. Development and validation of a rapid LC-MS/MS method for the detection of 182 novel psychoactive substances in whole blood. Drug Testing And Analysis. **https://doi.org/10.1002/dta.3170**

Gizer, I. R., Ficks, C., Waldman, I. D., 2009. Candidate gene studies of ADHD: a meta-analytic review. Human Genetics, 126, 51-90.

Goldman, L. S., Genel, M., Bezman, R. J., Slanetz, P. J., 1998. Diagnosis and treatment of attention-deficit/hyperactivity disorder in children and adolescents. Journal of the American Medical Association, 279, 1100-1107.

Gottlieb, S. 2001. Methylphenidate works by increasing dopamine levels. British Medical Journal, 322, 259-259.

Grand-Guillaume Perrenoud, A., Boccard, J., Veuthey, J.-L., Guillarme, D., 2012. Analysis of basic compounds by supercritical fluid chromatography: Attempts to

improve peak shape and maintain mass spectrometry compatibility. Journal of Chromatography A, 1262, 205-213.

Greenhill, L. L., Findling, R. L., Swanson, J. M., 2002a. A double-blind, placebo-controlled study of modified-release methylphenidate in children with attention-deficit/hyperactivity disorder. Pediatrics, 109, e39-e39.

Greenhill, L. L., Pliszka, S., Dulcan, M. K., 2002b. Practice parameter for the use of stimulant medications in the treatment of children, adolescents, and adults. Journal of the American Academy of Child & Adolescent Psychiatry, 41, 26S-49S.

Group, M. C., 2004. National Institute of Mental Health Multimodal Treatment Study of ADHD follow-up: changes in effectiveness and growth after the end of treatment. Pediatrics, 113, 762-769

Gualtieri, C. T., Johnson, L. G., 2008. Medications do not necessarily normalize cognition in ADHD patients. Journal of Attention Disorders, 11, 459-469

Hazell, P. L., Kohn, M. R., Dickson, R., Walton, R. J., Granger, R. E., van Wyk, G. W., 2011. Core ADHD symptom improvement with atomoxetine versus methylphenidate: a direct comparison meta-analysis. Journal of Attention Disorders, 15, 674-683.

Heal, D. J., Pierce, D. M., 2006. Methylphenidate and its isomers: their role in the treatment of attention-deficit hyperactivity disorder using a transdermal delivery system. CNS Drugs, 20, 713-738

Ho, J. H., Bailey, G. P., Archer, J. R., Dargan, P. I., Wood, D. M., 2015. Ethylphenidate: availability, patterns of use, and acute effects of this novel psychoactive substance. European Journal of Clinical Pharmacology, 71, 1185-1196.

Hodgkins, P., Shaw, M., Coghill, D., Hechtman, L., 2012. Amfetamine and methylphenidate medications for attention-deficit/ hyperactivity disorder: Complementary treatment options. European Child & Adolescent Psychiatry, 21, 477-492

Hurd, Y. L., Ungerstedt, U., 1989. In vivo neurochemical profile of dopamine uptake inhibitors and releasers in rat caudate-putamen. European Journal of Pharmacology, 166, 251-260.

Hutson, P.H., Pennick, M. and Secker, R. 2014. Preclinical pharmacokinetics, pharmacology and toxicology of lisdexamfetamine: A novel d-amphetamine pro-drug. Neuropharmacology, 87, 41-50.

Iden, C. R., Hungund, B. L., 1979. A chemical ionization selected ion monitoring assay for methylphenidate and ritalinic acid. Biomedical Mass Spectrometry, 6, 422-426

Jaeschke, R. R., Sujkowska, E., Sowa-Kućma, M., 2021. Methylphenidate for attention-deficit/hyperactivity disorder in adults: a narrative review. Psychopharmacology, 238, 2667-2691.

Jaffe, S. L., 1991. Intranasal abuse of prescribed methylphenidate by an alcohol and drug abusing adolescent with ADHD. Journal of the Americal Academy of Child and Adolescent Psychiatry, 30, 773-775

Jang, M., Kim, J., Shin, I., Kang, S., Choi, H., Yang, W., 2019. Simultaneous determination of methylphenidate and ritalinic acid in hair using LC–MS/MS. Forensic Science International, 294, 183-188.

Janowsky, A., Schweri, M. M., Berger, P., Long, R., Skolnick, P., Paul, S. M., 1985. The effects of surgical and chemical lesions on striatal [3H] threo-(±)-methylphenidate binding: correlation with [3H] dopamine uptake. European Journal of Pharmacology, 108, 187-191

Jasinski, D. and Krishnan, S. 2009. Abuse liability and safety of oral lisdexamfetamine dimesylate in individuals with a history of stimulant abuse. Journal of Psychopharmacology, 23, 419-427.

Jasinski, D. and Krishnan, S. 2009. Human pharmacology of intravenous lisdexamfetamine dimesylate: abuse liability in adult stimulant abusers. Journal of Psychopharmacology, 23, 410-418

Jiang-hai, L. U., Shan, W., Yang, Q. I. N., Jing, D., You-xuan, X. U., Mou-tian, W. U., 2009. Confirmation of Methylphenidate and Its Major Metabolite in Human Urine by Liquid Chromatography-Tandem Mass Spectrometry. Journal of Chinese Mass Spectrometry Society, 267-270.

Josefsson, M., Rydberg, I., 2011. Determination of methylphenidate and ritalinic acid in blood, plasma and oral fluid from adolescents and adults using protein precipitation and liquid chromatography tandem mass spectrometry—A method applied on clinical and forensic investigations. Journal of Pharmaceutical and Biomedical Analysis, 55, 1050-1059.

Kapur, A., 2020. Is Methylphenidate Beneficial and Safe in Pharmacological Cognitive Enhancement? CNS Drugs, 34, 1045-1062.

Kasabova, L. and Svinarov, D. 2015. Determination of Methylphenidate In Human Plasma By A Validated Lc-Ms/Ms Method. Clinical Therapeutics, 37, e62-e63.

Kehoe, W. A., 2001. Treatment of attention deficit hyperactivity disorder in children. Annals of Pharmacotherapy, 35, 1130-1134.

Kesner, R. P., Churchwell, J. C., 2011. An analysis of rat prefrontal cortex in mediating executive function. Neurobiology of Learning and Memory, 96, 417-431.

Kimko, H. C., Cross, J. T., Abernethy, D. R., 1999. Pharmacokinetics and clinical effectiveness of methylphenidate. Clinical Pharmacokinetics, 37, 457-470.

Kintz, P., Villain, M., 2010. Violence under the influence of methylphenidate as determined by hair analysis. Forensic Toxicology, 28, 115-118.

Kirkpatrick, Z.A. and Boyd, C.J. 2018. Stimulant Use Among Undergraduate Nursing Students. Journal of Addictions Nursing, 29.

Kollins, S. H., MacDonald, E. K., Rush, C. R., 2001. Assessing the abuse potential of methylphenidate in nonhuman and human subjects: a review. Pharmacology Biochemistry and Behavior, 68, 611-627.

Kooij, J. J. S., Bijlenga, D., Salerno, L., Jaeschke, R., Bitter, I., Balázs, J., Thome, J., Dom, G., Kasper, S., Nunes Filipe, C., Stes, S., Mohr, P., Leppämäki, S., Casas, M., Bobes, J., McCarthy, J. M., Richarte, V., Kjems Philipsen, A., Pehlivanidis, A., Niemela, A., Styr, B., Semerci, B., Bolea-Alamanac, B., Edvinsson, D., Baeyens, D.,

Wynchank, D., Sobanski, E., Philipsen, A., McNicholas, F., Caci, H., Mihailescu, I., Manor, I., Dobrescu, I., Saito, T., Krause, J., Fayyad, J., Ramos-Quiroga, J. A., Foeken, K., Rad, F., Adamou, M., Ohlmeier, M., Fitzgerald, M., Gill, M., Lensing, M., Motavalli Mukaddes, N., Brudkiewicz, P., Gustafsson, P., Tani, P., Oswald, P., Carpentier, P. J., De Rossi, P., Delorme, R., Markovska Simoska, S., Pallanti, S., Young, S., Bejerot, S., Lehtonen, T., Kustow, J., Müller-Sedgwick, U., Hirvikoski, T., Pironti, V., Ginsberg, Y., Félegyházy, Z., Garcia-Portilla, M. P., Asherson, P., 2019. Updated European Consensus Statement on diagnosis and treatment of adult ADHD. European Psychiatry, 56, 14-34.

Koehm, M., Kauert, G.F. and Toennes, S.W. 2010. Influence of ethanol on the pharmacokinetics of methylphenidate's metabolites ritalinic acid and ethylphenidate. Arzneimittelforschung, 60, 238-44.

Krishnan, S.M., Pennick, M. and Stark, J.G. 2008. Metabolism, distribution and elimination of lisdexamfetamine dimesylate. Clinical Drug Investigation, 28, 745-755.

Kroutil, L. A., Van Brunt, D. L., Herman-Stahl, M. A., Heller, D. C., Bray, R. M., Penne, M. A., 2006. Nonmedical use of prescription stimulants in the United States. Drug and Alcohol Dependence, 84, 135-143.

Krueger, J., Sachs, H., Musshoff, F., Dame, T., Schaeper, J., Schwerer, M., Graw, M., Roider, G., 2014. First detection of ethylphenidate in human fatalities after ethylphenidate intake. Forensic Science International, 243, 126-129.

Kwon, W., 서승일, 인문교, 김진영, 2014. Simultaneous Determination of Methylphenidate, Amphetamine and their Metabolites in Urine using Direct Injection Liquid Chromatography-Tandem Mass Spectrometry. Mass Spectrometry Letters, 5, 104-109.

Lange, K. W., Reichl, S., Lange, K. M., Tucha, L., Tucha, O., 2010. The history of attention deficit hyperactivity disorder. Attention Deficit and Hyperactivity Disorders, 2, 241-255.

Leis, H. J., Schütz, H., Windischhofer, W., 2011. Quantitative determination of methylphenidate in plasma by gas chromatography negative ion chemical ionisation mass spectrometry using o-(pentafluorobenzyloxycarbonyl)-benzoyl derivatives. Analytical & Bioanalytical Chemistry, 400, 2663-2670.

Lesellier, E. and West, C. 2015. The many faces of packed column supercritical fluid chromatography – A critical review. Journal of Chromatography A, 1382, 2-46.

LeVasseur, N. L., Zhu, H.-J., Markowitz, J. S., DeVane, C. L., Patrick, K. S., 2008. Enantiospecific gas chromatographic–mass spectrometric analysis of urinary methylphenidate: Implications for phenotyping. Journal of Chromatography B, 862, 140-149.

Levi, G., Raiteri, M., 1993. Carrier-mediated release of neurotransmitters. Trends in Neurosciences, 16, 415-419.

Lin, S.-N., Andrenyak, D. M., Moody, D. E., Foltz, R. L., 1999. Enantioselective Gas Chromatography-Negative Ion Chemical Ionization Mass Spectrometry for Methylphenidate in Human Plasma. Journal of Analytical Toxicology, 23, 524-530.

Loe, I. M., Feldman, H. M., 2007. Academic and educational outcomes of children with ADHD. Journal of Pediatric Psychology, 32, 643-654.

Logan, B. K., D'Orazio, A. L., Mohr, A. L. A., Limoges, J. F., Miles, A. K., Scarneo, C. E., Kerrigan, S., Liddicoat, L. J., Scott, K. S., Huestis, M. A., 2018. Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities-2017 Update. Journal of Analytical Toxicology, 42, 63-68.

Low, K. G., Gendaszek, A. E., 2002. Illicit use of psychostimulants among college students: A preliminary study. Psychology, Health & Medicine, 7, 283-287.

Luo, X.-M., Ding, L., Gu, X., Jiang, L.-Y., Dong, X., 2014. [LC-MS/MS assay of methylphenidate: stability and pharmacokinetics in human]. Yao xue xue bao = Acta pharmaceutica Sinica, 49, 83-88.

Mannuzza, S., Klein, R. G., Truong, N. L., Moulton III, P. D., John L, Roizen, E. R., Howell, K. H., Castellanos, F. X., 2008. Age of methylphenidate treatment initiation in children with ADHD and later substance abuse: prospective follow-up into adulthood. American Journal of Psychiatry, 165, 604-609.

Marchei, E., Farrè, M., Pellegrini, M., Rossi, S., García-Algar, Ó., Vall, O., et al. 2009. Liquid chromatography–electrospray ionization mass spectrometry determination of methylphenidate and ritalinic acid in conventional and non-conventional biological matrices. Journal of Pharmaceutical and Biomedical Analysis, 49, 434-439.

Markowitz, J. S., DeVane, C. L., Boulton, D. W., Nahas, Z., Risch, S. C., Diamond, F., Patrick, K. S., 2000. Ethylphenidate Formation in Human Subjects after the Administration of a Single Dose of Methylphenidate and Ethanol. Drug Metabolism and Disposition, 28, 620.

Markowitz, J. S., DeVane, C. L., Pestreich, L. K., Patrick, K. S., Muniz, R., 2006. A comprehensive in vitro screening of d-, l-, and dl-threo-methylphenidate: an exploratory study. Journal of Child & Adolescent Psychopharmacology, 16, 687-698.

Markowitz, J. S., Logan, B. K., Diamond, F., Patrick, K. S., 1999. Detection of the Novel Metabolite Ethylphenidate After Methylphenidate Overdose With Alcohol Coingestion. Journal of Clinical Psychopharmacology, 19.

Markowitz, J. S., Patrick, K. S., 2008. Differential pharmacokinetics and pharmacodynamics of methylphenidate enantiomers: does chirality matter? Journal of Clinical Psychopharmacology, 28, S54-S61.

Markowitz, J. S., Patrick, K. S., 2013. Ethylphenidate: From biomarker to designer drug. Mental Health Clinician, 3, 318-320.

Markowitz, J. S., Straughn, A. B., Patrick, K. S., 2003a. Advances in the Pharmacotherapy of Attention-Deficit-Hyperactivity Disorder: Focus on Methylphenidate Formulations. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 23, 1281-1299.

Markowitz, J. S., Straughn, A. B., Patrick, K. S., DeVane, C. L., Pestreich, L., Lee, J., Wang, Y., Muniz, R., 2003b. Pharmacokinetics of Methylphenidate After Oral

Administration of Two Modified-Release Formulations in Healthy Adults. Clinical Pharmacokinetics, 42, 393-401.

Maskell, P. D., Smith, P. R., Cole, R., Hikin, L., Morley, S. R., 2016. Seven fatalities associated with ethylphenidate. Forensic Science International, 265, 70-74.

Maul, J., Advokat, C., 2013. Stimulant medications for attention-deficit/hyperactivity disorder (ADHD) improve memory of emotional stimuli in ADHD-diagnosed college students. Pharmacology Biochemistry and Behavior, 105, 58-62.

McCabe, S. E., Boyd, C. J., 2005. Sources of prescription drugs for illicit use. Addictive Behaviors, 30, 1342-1350.

McCabe, S. E., Knight, J. R., Teter, C. J., Wechsler, H., 2005. Non-medical use of prescription stimulants among US college students: prevalence and correlates from a national survey. Addiction, 100, 96-106.

McCabe, S. E., Teter, C. J., Boyd, C. J., 2004. The Use, Misuse and Diversion of Prescription Stimulants Among Middle and High School Students. Substance Use & Misuse, 39, 1095-1116

Midha, K., McKay, G., Rawson, M., Korchinski, E., Hubbard, J., 2001. Effects of food on the pharmacokinetics of methylphenidate. Pharmaceutical Research, 18, 1185-1189

Ministry of Health, D., 2013a. Bekendtgørelse om ændring af bekendtgørelse om euforiserende stoffer (Order amending the Order on drugs).

Ministry of Health, G., 2013b. Siebenundzwanzigste Verordnung zur Änderung betäubungsmittel-rechtlicher Vorschriften (Twenty-Seventh Regulation amendment of the Narcotics Legislation).

Ministry of Social Affairs, S., Förordning (1992:1554) om kontroll av narkotika (Regulation (1992: 1554) on control of narcotics).

Modesto-Lowe, V., Meyer, A., Soovajian, V., 2012. A clinican's guide to adult attention-deficit hyperactivity disorder. Connecticut Medicine, 76.

Mulet, C. T., Arroyo-Mora, L. E., Leon, L. A., Gnagy, E., DeCaprio, A. P., 2018. Rapid quantitative analysis of methylphenidate and ritalinic acid in oral fluid by liquid chromatography triple quadrupole mass spectrometry (LC-QqQ-MS). Journal of Chromatography B, 1092, 313-319.

Nelson, J. M., Liebel, S. W., 2018. Anxiety and depression among college students with attention-deficit/hyperactivity disorder (ADHD): Cross-informant, sex, and subtype differences. Journal of American College Health, 66, 123-132.

Nováková, L., Grand-Guillaume Perrenoud, A., Francois, I., West, C., Lesellier, E., Guillarme, D., 2014. Modern analytical supercritical fluid chromatography using columns packed with sub-2μm particles: A tutorial. Analytica Chimica Acta, 824, 18-35.

Nutt, D., King, L. A., Saulsbury, W., Blakemore, C., 2007. Development of a rational scale to assess the harm of drugs of potential misuse. The Lancet, 369, 1047-1053.

Nzekoue, F. K., Agostini, M., Verboni, M., Renzoni, C., Alfieri, L., Barocci, S., Ricciutelli, M., Caprioli, G., Lucarini, S., 2021. A comprehensive UHPLC–MS/MS screening method for the analysis of 98 New Psychoactive Substances and related compounds in human hair. Journal of Pharmaceutical and Biomedical Analysis, 205, 114310.

Parekh, P. K., Ozburn, A. R., McClung, C. A., 2015. Circadian clock genes: Effects on dopamine, reward and addiction. Alcohol, 49, 341-349.

Parks, C., McKeown, D., Torrance, H. J., 2015. A review of ethylphenidate in deaths in east and west Scotland. Forensic Science International, 257, 203-208.

Paterson, S. M., Moore, G. A., Florkowski, C. M., George, P. M., 2012. Determination of methylphenidate and its metabolite ritalinic acid in urine by liquid chromatography/tandem mass spectrometry. Journal of Chromatography , 881-882, 20-26.

Patrick, K.S., Caldwell, R.W., Ferris, R.M. and Breese, G.R. 1987. Pharmacology of the enantiomers of threo-methylphenidate. J Pharmacol Exp Ther 241:152-158.

Patrick, K. S., Corbin, T. R., Murphy, C. E., 2014. Ethylphenidate as a Selective Dopaminergic Agonist and Methylphenidate–Ethanol Transesterification Biomarker. Journal of Pharmaceutical Sciences, 103, 3834-3842

Patrick, K. S., González, M. A., Straughn, A. B., Markowitz, J. S., 2005a. New methylphenidate formulations for the treatment of attention-deficit/hyperactivity disorder. Expert Opinion on Drug Delivery, 2, 121-143.

Patrick, K.S. and Markowitz, J.S. 1997. Pharmacology of methylphenidate, amphetamine enantiomers and pemoline in attention-deficit hyperactivity disorder. Human Psychopharmogy: Clinical and Experimental, 12:527-546.

Patrick, K. S., Straughn, A. B., Minhinnett, R. R., Yeatts, S. D., Herrin, A. E., DeVane, C. L., Malcolm, R., Janis, G. C., Markowitz, J. S., 2007. Influence of Ethanol and Gender on Methylphenidate Pharmacokinetics and Pharmacodynamics. Clinical Pharmacology and Therapeutics, 81, 346-353.

Patrick, K. S., Straughn, A. B., Reeves, O. T., Bernstein, H., Bell, G. H., Anderson, E. R., Malcolm, R. J., 2013. Differential Influences of Ethanol on Early Exposure to Racemic Methylphenidate Compared with Dexmethylphenidate in Humans. Drug Metabolism and Disposition, 41, 197-205.

Patrick, K. S., Williard, R. L., VanWert, A. L., Dowd, J. J., Oatis, J. E., Middaugh, L. D., 2005b. Synthesis and pharmacology of ethylphenidate enantiomers: the human transesterification metabolite of methylphenidate and ethanol. Journal of Medicinal Chemistry, 48, 2876-2881.

Pauk, V. and Lemr, K. 2018. Forensic applications of supercritical fluid chromatography – mass spectrometry. Journal of Chromatography B, 1086, 184-196.

Pennick, M. 2010. Absorption of lisdexamfetamine dimesylate and its enzymatic conversion to d-amphetamine. Neuropsychiatric Disease and Treatment, 6, 317-327.

Pennick, M. 2013. Metabolism of the prodrug lisdexamfetamine dimesylate in human red blood cells from normal and sickle cell disease donors. Journal of Drug Assessment, 2, 17-20.

Peters, F.T., Drummer, O.H. and Musshoff, F. 2007. Validation of new methods. Forensic Science International, 165, 216-24.

Pliszka, S. R., 2005. The neuropsychopharmacology of attention-deficit/hyperactivity disorder. Biological Psychiatry, 57, 1385-1390.

Pliszka, S. R., 2007. Pharmacologic treatment of attention-deficit/hyperactivity disorder: efficacy, safety and mechanisms of action. Neuropsychology Review, 17, 61-72.

Preiskorn, J., Studer, S., Rauh, R., Lukačin, R., Geffert, C., Fleischhaker, C., et al. 2018. Inter- and Intraindividual Variation of Methylphenidate Concentrations in Serum and Saliva of Patients with Attention-Deficit/Hyperactivity Disorder. Therapeutic Drug Monitoring, 40:435-442

Polanczyk, G. V., Salum, G. A., Sugaya, L. S., Caye, A., Rohde, L. A., 2015. Annual research review: A meta-analysis of the worldwide prevalence of mental disorders in children and adolescents. Journal of Child Psychology and Psychiatry, 56, 345-365.

Portoghese, P. S., Malspeis, L., 1961. Relative Hydrolytic Rates of Certain Alkyl (b) dl-α-(2-Piperidyl)-phenylacetates. Journal of Pharmaceutical Sciences, 50, 494-501.

Potts, B. D., Martin, C. A., Vore, M., 1984. Gas-chromatographic quantification of methylphenidate in plasma with use of solid-phase extraction and nitrogen-sensitive detection. Clinical Chemistry, 30, 1374-1377.

Poulton, A., 2005. Growth on stimulant medication; clarifying the confusion: a review. Archives of Disease in Childhood, 90, 801-806.

Radcliffe, C., Maguire, K., Lockwood, B., 2000. Applications of supercritical fluid extraction and chromatography in forensic science. Journal of Biochemical and Biophysical Methods, 43, 261-272.

Raman, S. R., Man, K. K., Bahmanyar, S., Berard, A., Bilder, S., Boukhris, T., Bushnell, G., Crystal, S., Furu, K., KaoYang, Y.-H., 2018. Trends in attention-deficit hyperactivity disorder medication use: a retrospective observational study using population-based databases. The Lancet Psychiatry, 5, 824-835.

Ramos, L., Bakhtiar, R., Majumdar, T., Hayes, M., Tse, F., 1999. Liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry enantiomeric separation of dl-threo-methylphenidate, (Ritalin®) using a macrocyclic antibiotic as the chiral selector. Rapid Communications in Mass Spectrometry, 13, 2054-2062.

Ramos, L., Bakhtiar, R. and Tse, F.L.S. 2000. Liquid-liquid extraction using 96-well plate format in conjunction with liquid chromatography/tandem mass spectrometry for quantitative determination of methylphenidate (Ritalin®) in human plasma. Rapid Communications in Mass Spectrometry, 14, 740-745.

Reed, G. M., First, M. B., Kogan, C. S., Hyman, S. E., Gureje, O., Gaebel, W., Maj, M., Stein, D. J., Maercker, A., Tyrer, P., 2019. Innovations and changes in the ICD-11 classification of mental, behavioural and neurodevelopmental disorders. World Psychiatry, 18, 3-19.

Richeval, C., Gish, A., Hakim, F., Nachon-Phanithavong, M., Wiart, J.-F., Humbert, L., et al. 2021. Prevalence of New Psychoactive Substances in Oral Fluid Samples from

French Drivers: A Longitudinal Survey (2016–2020). Journal of Analytical Toxicology, 45, e20-e21.

Riddle, E. L., Hanson, G. R., Fleckenstein, A. E., 2007. Therapeutic doses of amphetamine and)methylphenidate selectively redistribute the vesicular monoamine transporter-2. European Journal of Pharmacology, 571, 25-28.

Ritz, M.C., Lamb, R.J., Goldberg, S.R. and Kuhar, M.J. 1987. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science, 237, 1219-1223.

Robbins, T. W., 2003. Dopamine and cognition. Current Opinion in Neurology, 16, S1-S2.

Roesch, B., Corcoran, M.E., Fetterolf, J., Haffey, M., Martin, P., Preston, P., et al. 2013. Pharmacokinetics of coadministered guanfacine extended release and lisdexamfetamine dimesylate. Drugs in R&D, 13, 119-128.

Ross, M. M., Arria, A. M., Brown, J. P., Mullins, C. D., Schiffman, J., Simoni-Wastila, L., dosReis, S., 2018. College students' perceived benefit-to-risk tradeoffs for nonmedical use of prescription stimulants: Implications for intervention designs. Addictive Behaviors, 79, 45-51.

Rowley, H., Kulkarni, R., Gosden, J., Brammer, R., Hackett, D. and Heal, D. 2012. Lisdexamfetamine and immediate release d-amfetamine–Differences in pharmacokinetic/pharmacodynamic relationships revealed by striatal microdialysis in freely-moving rats with simultaneous determination of plasma drug concentrations and locomotor activity. Neuropharmacology, 63, 1064-1074.

Saito, M. 2013. History of supercritical fluid chromatography: Instrumental development. Journal of Bioscience and Bioengineering, 115, 590-599.

Saito, K., Saito, R., Ito, R., 2021. Determination of methamphetamine and methylphenidate in urine by liquid chromatography/time-of-flight mass spectrometry coupled with solid-phase dispersive extraction, and its pharmacokinetic application. Forensic Chemistry, 24, 100334.

Sandoval, V., Riddle, E. L., Hanson, G. R., Fleckenstein, A. E., 2002. Methylphenidate redistributes vesicular monoamine transporter-2: role of dopamine receptors. Journal of Neuroscience, 22, 8705-8710.

Schulenberg, J., Johnston, L., O'Malley, P., Bachman, J., Miech, R., Patrick, M., 2017. College Students & Adults Ages 19-55. National Survey Results on Drug Use 1975-2017. Monitoring the Future, 476.

Schweri, M. M., Skolnick, P., Rafferty, M. F., Rice, K. C., Janowsky, A. J., Paul, S. M., 1985. [3H]Threo-(±)-Methylphenidate Binding to 3,4-Dihydroxyphenylethylamine Uptake Sites in Corpus Striatum: Correlation with the Stimulant Properties of Ritalinic Acid Esters. Journal of Neurochemistry, 45, 1062-1070.

Schulz, M., Iwersen-Bergmann, S., Andresen, H. and Schmoldt, A. 2012. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. Critical Care, 16, R136.

Seçilir, A., Schrier, L., Bijleveld, Y. A., Toersche, J. H., Jorjani, S., Burggraaf, J., van Gerven, J., Mathôt, R. A. A., 2013. Determination of methylphenidate in plasma and

saliva by liquid chromatography/tandem mass spectrometry. Journal of Chromatography B, 923-924, 22-28.

Seiden, L. S., Sabol, K. E., Ricaurte, G. A., 1993. Amphetamine: effects on catecholamine systems and behavior. Annual Review of Pharmacology and Toxicology, 33, 639-676.

Sharma, A., Couture, J., 2013. A Review of the Pathophysiology, Etiology, and Treatment of Attention-Deficit Hyperactivity Disorder (ADHD). Annals of Pharmacotherapy, 48, 209-225.

Sie, S.T., Van Beersum, W. and Rijnders, G.W.A. (1966) High-Pressure Gas Chromatography and Chromatography with Supercritical Fluids. I. The Effect of Pressure on Partition Coefficients in Gas-Liquid Chromatography with Carbon Dioxide as a Carrier Gas. Separation Science, 1, 459-490.

Silva, M.R., Andrade, F.N., Fumes, B.H. and Lanças, F.M. 2015. Unified chromatography: Fundamentals, instrumentation and applications. Journal of Separation Science, 38, 3071-3083

Smith, M. E., Farah, M. J., 2011. Are prescription stimulants “smart pills”? The epidemiology and cognitive neuroscience of prescription stimulant use by normal healthy individuals. Psychological Bulletin, 137, 717.

Smith, C.R. and Swortwood, M.J. 2021. Short- and Long-Term Stability of Methylphenidate and Its Metabolites in Blood. Journal of Analytical Toxicology, 45, 863-869.

Soussan, C., Kjellgren, A., 2015. “Chasing the High” – Experiences of Ethylphenidate as Described on International Internet Forums. Substance Abuse: Research and Treatment, 9, SART.S22495.

Spencer, T., Biederman, J., Wilens, T., 2004. Nonstimulant treatment of adult attention-deficit/hyperactivity disorder. The Psychiatric Clinics of North America, 27, 373-383.

Spencer, T. J., Wilens, T. E., Biederman, J., Weisler, R. H., Read, S. C., Pratt, R., 2006. Efficacy and safety of mixed amphetamine salts extended release (Adderall XR) in the management of attention-deficit/hyperactivity disorder in adolescent patients: a 4-week, randomized, double-blind, placebo-controlled, parallel-group study. Clinical Therapeutics, 28, 266-279.

Srinivas, N.R., Hubbard, J.W., Korchinski, E.D. and Midha, K.K. 1992. Stereoselective Urinary Pharmacokinetics of dl-threo-Methylphenidate and Its Major Metabolite in Humans. Journal of Pharmaceutical Sciences, 81, 747-749.

Srinivas, N.R., Hubbard, J.W., Korchinski, E.D. and Midha, K.K. 1993. Enantioselective Pharmacokinetics of dl-threo-Methylphenidate in Humans. Pharmaceutical Research, 10, 14-21.

Srinivas, N.R., Hubbard, J.W., McKay, G., Hawes, E.M. and Midha, K.K. 1991. In vitro hydrolysis of RR,SS-threo-methylphenidate by blood esterases-- differential and enantioselective interspecies variability. Chirality, 3, 99-103.

Steven Pliszka, A. W. G. o. Q. I., 2007. Practice parameter for the assessment and treatment of children and adolescents with attention-deficit/hyperactivity disorder. Journal of the American Academy of Child and Adolescent Psychiatry, 46, 894-921.

Stevens, J. R., George, R. A., Fusillo, S., Stern, T. A., Wilens, T. E., 2010. Plasma methylphenidate concentrations in youths treated with high-dose osmotic release oral system formulation. Journal of Child and Adolescent Psychopharmacology, 20, 49-54.

Stevens, T., Sangkuhl, K., Brown, J. T., Altman, R. B., Klein, T. E., 2019. PharmGKB summary: methylphenidate pathway, pharmacokinetics/pharmacodynamics. Pharmacogenetics and Genomics, 29, 136.

Studer, S., Burghardt, S., Fleischhaker, C., Schulz, E., Clement, H.W. and Lukačin, R. 2014. Methylphenidate and ritalinic acid determination in serum and saliva from patients with attention deficit hyperactivity disorder. Pharmacopsychiatry, 47, A4

Studer, S., Preiskorn, J., Lukacin, R., Geffert, C., Fleischhaker, C., Clement, H.W., et al. 2016. Inter- and intraindividual variations of methylphenidate in serum and oral fluid of ADHS patients. Pharmacopsychiatry, 26, P12.

Sun, Z., Murry, D. J., Sanghani, S. P., Davis, W. I., Kedishvili, N. Y., Zou, Q., Hurley, T. D., Bosron, W. F., 2004. Methylphenidate is stereoselectively hydrolyzed by human carboxylesterase CES1A1. Journal of Pharmacology and Experimental Therapeutics, 310, 469-476.

Swanson, J., Greenhill, L., Wigal, T., Kollins, S., Stehli, A., Davies, M., Chuang, S., Vitiello, B., Skrobala, A., Posner, K., 2006. Stimulant-related reductions of growth

rates in the PATS. Journal of the American Academy of Child & Adolescent Psychiatry, 45, 1304-1313.

Swanson, J. M., Cantwell, D., Lerner, M., McBurnett, K., Hanna, G., 1991. Effects of stimulant medication on learning in children with ADHD. Journal of Learning Disabilities, 24, 219-230.

Szporny, L. and Görög, P. 1961. Investigations into the correlations between monoamine oxidase inhibition and other effects due to methylphenydate and its stereoisomers. Biochemistry, 8:263-268.

Tarafder, A. 2016. Metamorphosis of supercritical fluid chromatography to SFC: An Overview. TrAC Trends in Analytical Chemistry, 81, 3-10.

Thomsen, R., Rasmussen, H. B., Linnet, K., the, I. C., 2012. Enantioselective Determination of Methylphenidate and Ritalinic Acid in Whole Blood from Forensic Cases Using Automated Solid-Phase Extraction and Liquid Chromatography–Tandem Mass Spectrometry. Journal of Analytical Toxicology, 36, 560-568.

Thomson, M. R., Dowd, J. J., Markowitz, J., Devane, C., Patrick, K. S., 2002. Enantioselective transesterification of methylphenidate to ethylphenidate after coadministration with ethanol. The Journal of Clinical Pharmacology, 42, 1069-1069.

Trent, S., Davies, W., 2012. The influence of sex-linked genetic mechanisms on attention and impulsivity. Biological Psychology, 89, 1-13.

Tripp, G., Wickens, J. R., 2009. Neurobiology of ADHD. Neuropharmacology, 57, 579-589.

UK Chemical Resarch, Thread - Ethylphenidate. https://www.ukchemicalresearch.org/thread-ethylphenidate (Accessed April 2021)

Upadhyaya, H. P., Rose, K., Wang, W., O'Rourke, K., Sullivan, B., Deas, D., Brady, K. T., 2005. Attention-deficit/hyperactivity disorder, medication treatment, and substance use patterns among adolescents and young adults. Journal of Child & Adolescent Psychopharmacology, 15, 799-809.

Valentine, J. L., Middleton, R., 2000. GC-MS identification of sympathomimetic amine drugs in urine: rapid methodology applicable for emergency clinical toxicology. Journal of Analytical Toxicology, 24, 211-222.

Volkow, N. D., Ding, Y.-S., Fowler, J. S., Wang, G.-J., Logan, J., Gatley, J. S., Dewey, S., Ashby, C., Liebermann, J., Hitzemann, R., 1995. Is methylphenidate like cocaine?: Studies on their pharmacokinetics and distribution in the human brain. Archives of General Psychiatry, 52, 456-463.

Volkow, N. D., Swanson, J. M., 2013. Adult attention deficit–hyperactivity disorder. New England Journal of Medicine, 369, 1935-1944.

Volkow, N. D., Wang, G.-J., Fowler, J. S., Gatley, S. J., Logan, J., Ding, Y.-S., Hitzemann, R., Pappas, N., 1998. Dopamine transporter occupancies in the human brain induced by therapeutic doses of oral methylphenidate. American Journal of Psychiatry, 155, 1325-1331.

Volkow, N. D., Wang, G.-J., Fowler, J. S., Telang, F., Maynard, L., Logan, J., Gatley, S. J., Pappas, N., Wong, C., Vaska, P., 2004. Evidence that methylphenidate enhances

the saliency of a mathematical task by increasing dopamine in the human brain. American Journal of Psychiatry, 161, 1173-1180.

Wall, S. C., Gu, H., Rudnick, G., 1995. Biogenic amine flux mediated by cloned transporters stably expressed in cultured cell lines: amphetamine specificity for inhibition and efflux. Molecular Pharmacology, 47, 544-550.

Wang, S.-M., Ling, Y.-C., Giang, Y.-S., 2003. Forensic applications of supercritical fluid extraction and chromatography. Forensic Science Journal, 2.

Wargin, W., Patrick, K., Kilts, C., Gualtieri, C.T., Ellington, K., Mueller, R.A., et al. (1983) Pharmacokinetics of methylphenidate in man, rat and monkey. Journal of Pharmacology and Experimental Therapeutics, 226, 382-386

Waxmonsky, J. G., 2005. Nonstimulant therapies for attention-deficit hyperactivity disorder (ADHD) in children and adults. Essential Psychopharmacology, 6, 262-276.

Wenthur, C. J., 2016. Classics in Chemical Neuroscience: Methylphenidate. ACS Chemical Neuroscience, 7, 1030-1040.

Wernicke, J., Kratochvil, C. J., 2002. Safety profile of atomoxetine in the treatment of children and adolescents with ADHD. Journal of Clinical Psychiatry, 63, 50-55.

West, C. 2019. Recent trends in chiral supercritical fluid chromatography. TrAC Trends in Analytical Chemistry, 120, 115648.

White, B. P., Becker-Blease, K. A., Grace-Bishop, K., 2006. Stimulant medication use, misuse, and abuse in an undergraduate and graduate student sample. Journal of American College Health, 54, 261-268.

Wilens, T. E., 2007. The nature of the relationship between attention-deficit/hyperactivity disorder and substance use. Journal of Clinical Psychiatry, 68 Suppl 11, 4-8.

Wilens, T. E., Adler, L. A., Adams, J., Sgambati, S., Rotrosen, J., Sawtelle, R., Utzinger, L., Fusillo, S., 2008. Misuse and diversion of stimulants prescribed for ADHD: a systematic review of the literature. Journal of the American Academy of Child and Adolescent Psychiatry, 47, 21-31.

Wilens, T. E., Gignac, M., Swezey, A., Monuteaux, M. C., Biederman, J., 2006a. Characteristics of adolescents and young adults with ADHD who divert or misuse their prescribed medications. Journal of the American Academy of Child and Adolescent Psychiatry, 45, 408-414.

Wilens, T. E., McBurnett, K., Bukstein, O., McGough, J., Greenhill, L., Lerner, M., Stein, M. A., Conners, C. K., Duby, J., Newcorn, J., 2006b. Multisite controlled study of OROS methylphenidate in the treatment of adolescents with attention-deficit/hyperactivity disorder. Archives of Pediatrics & Adolescent Medicine, 160, 82-90.

Wilens, T.E., Morrison, N.R. and Prince, J. 2011. An update on the pharmacotherapy of attention-deficit/hyperactivity disorder in adults. Expert Review on Neurotherapeutics, 11, 1443-65.

Williard, R., Middaugh, L., Zhu, H.-J., Patrick, K., 2007. Methylphenidate and its ethanol transesterification metabolite ethylphenidate: Brain disposition, monoamine transporters and motor activity. Behavioural Pharmacology, 18, 39-51.

Wilens, T.E., Morrison, N.R. and Prince, J. 2011. An update on the pharmacotherapy of attention-deficit/hyperactivity disorder in adults. Expert Review on Neurotherapeutics, 11, 1443-65.

Wilson, W., 1995. Supercritical Fluid Chromatography. Journal of Chromatography A, 691, 246.

Wolraich, M. L., Doffing, M. A., 2004. Pharmacokinetic Considerations in the Treatment of Attention-Deficit Hyperactivity Disorder with Methylphenidate. CNS Drugs, 18, 243-250.

Wu, L.-T., Pilowsky, D.J., Schlenger, W.E. and Galvin, D.M. 2007. Misuse of methamphetamine and prescription stimulants among youths and young adults in the community. Drug and Alcohol Dependence, 89, 195-205.

Zancanaro, I., Limberger, R.P., Bohel, P.O., dos Santos, M.K., De Boni, R.B., Pechansky, F., et al. 2012. Prescription and illicit psychoactive drugs in oral fluid—LC–MS/MS method development and analysis of samples from Brazilian drivers. Forensic Science International, 223, 208-216

Zhang, C., Luo, H., Wu, Y., Zhang, J., Zhang, F., Lin, G., Wang, H., 2016. Development and validation of an UFLC-MS/MS method for enantioselectivity determination of d,l-thero-methylphenidate, d,l-thero-ethylphenidate and d,l-thero-ritalinic acid in rat plasma and its application to pharmacokinetic study. Journal of Chromatography B, 1011, 45-52.

Zhang, J., Deng, Y., Fang, J., McKay, G., 2003. Enantioselective analysis of ritalinic acids in biological samples by using a protein-based chiral stationary phase. Pharmaceutical Research, 20, 1881-1884.

Zhu, H.-J., Patrick, K. S., Markowitz, J. S., 2011. Enantiospecific determination of dl-methylphenidate and dl-ethylphenidate in plasma by liquid chromatography–tandem mass spectrometry: Application to human ethanol interactions. Journal of Chromatography B, 879, 783-788.

Zhu, H.-J., Patrick, K. S., Straughn, A. B., Reeves III, O. T., Bernstein, H., Shi, J., Johnson, H. J., Knight, J. M., Smith, A. T., Malcolm, R. J., 2017. Ethanol interactions with dexmethylphenidate and dl-methylphenidate spheroidal oral drug absorption systems in healthy volunteers. Journal of Clinical Psychopharmacology, 37, 419.

Zhu, H.-J., Wang, J.-S., Patrick, K. S., Donovan, J. L., Devane, C. L., Markowitz, J.S. 2007. A novel HPLC fluoroescence method for the quantification of methylpheninate in human plasma. Journal of Chromatography B, 858, 91-95.

APPENDIX A

Sam Houston STATE UNIVERSITY
A Member of The Texas State University System

Date: Sep 17, 2019 9:11 PM CDT

TO: Christina Smith Madeline Swortwood
FROM: SHSU IRB
PROJECT TITLE: ADHD Medication Abuse and Misuse and Its Implications on College Students - Sample Collection
PROTOCOL #: IRB-2019-225
SUBMISSION TYPE: Initial
ACTION: Approved
DECISION DATE: September 16, 2019
ADMINITRATIVE CHECK-IN DATE: September 16, 2020
EXPEDITED REVIEW CATEGORY: 3. Prospective collection of biological specimens for research purposes by noninvasive means.
7. Research on individual or group characteristics or behavior (including, but not limited to, research on perception, cognition, motivation, identity, language, communication, cultural beliefs or practices, and social behavior) or research employing survey, interview, oral history, focus group, program evaluation, human factors evaluation, or quality assurance methodologies.

Greetings,

The above-referenced submission has been reviewed by the IRB and it has been Approved. Because this study received expedited review and the IRB determined that a renewal submission is not needed, this decision does not necessarily expire; however, you will be receiving an email notification on the anniversary of this study approval, which will be on September 16, 2020 (**NOTE:** please review the reminder information below regarding Study Administrative Check-In). This study approval is based on an appropriate risk/benefit ratio and a project design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

Since Cayuse IRB does not currently possess the ability to provide a "stamp of approval" on any recruitment or consent documentation, it is the strong recommendation of this office to please include the following approval language in the footer of those recruitment and consent documents: IRB-2019-225/September 16, 2019/September 16, 2020.

Please remember that informed consent is a process beginning with a description of the project and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the project via a dialogue between the researcher and research participant. Federal regulations require each participant receive a

copy of the signed consent document.

Modifications: Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please submit a Modification Submission through Cayuse IRB for this procedure.

Incidents: All UNANTICIPATED PROBLEMS involving risks to subjects or others and SERIOUS and UNEXPECTED adverse events must be reported promptly to this office. Please submit an Incident Submission through Cayuse IRB for this procedure. All Department of Health and Human Services and sponsor reporting requirements should also be followed.

Study Administrative Check-In: Based on the risks, this project does not require renewal. Rather, you are required to administratively check in with the IRB on an annual basis. September 16, 2020 is the anniversary of the review of your protocol. The following are the conditions of the IRB approval for IRB-2019-225 ADHD Medication Abuse and Misuse and Its Implications on College Students - Sample Collection.

1. When this project is finished or terminated, a **Closure submission** is required.

2. Changes to the approved protocol require prior board approval (**NOTE:** see the directive above related to **Modifications**).

3. Human subjects training is required to be kept current at citiprogram.org by renewing training every 5 years.

Please note that all research records should be retained for a minimum of three years after the completion of the project. If you have any questions, please contact the Sharla Miles at 936-294-4875 or irb@shsu.edu. Please include your protocol number in all correspondence with this committee.

Sincerely,

Donna M. Desforges, Ph.D.
Chair, Committee for the Protection of Human Subjects
PHSC-IRB

APPENDIX B

Informed Consent

My name is Christina Smith, and I am a doctoral student of the Department of Forensic Sciences at Sam Houston State University. I would like to take this opportunity to invite you to participate in a research study to examine the use of ADHD medications on college campuses using oral fluid (saliva). I am conducting this research under the direction of Dr. Madeleine Swortwood. We hope that data from this research will inform us about drug use trends. You have been asked to participate in the research because you are a SHSU student and we are looking at the use of ADHD medications among a college population.

The research is relatively straightforward, and we do not expect the research to pose any risk to any of the volunteer participants. If you consent to participate in this research, you will be asked to swab your mouth with a small pad to collect oral fluid (saliva). Any data obtained from you will only be used for the purpose of generalizing drug trends. Under no circumstances will you or any other participants who participated in this research be identified. In addition, your data will remain confidential. This research will require about 15 minutes of your time. Participants will be paid or otherwise compensated for their participation in this project by small incentives such as blue books, scantrons, pens or pencils, or candy.

Your participation in this research is voluntary. Your decision whether or not to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled. If you have any questions, please feel free to ask me using the contact information below. If you are interested, the results of this study will be available at the conclusion of the project.

If you have any questions about this research, please feel free to contact me, Christina Smith, or Dr. Madeleine Swortwood. If you have questions or concerns about your rights

as research participants, please contact Sharla Miles, Office of Research and Sponsored Programs, using her contact information below.

Christina Smith *Dept. of Forensic Science* *Sam Houston State University* *Huntsville, TX 77341* *Phone: (936) 294-4319* *E-mail: crs040@shsu.edu*	*Dr. Madeleine Swortwood* *Dept. of Forensic Science* *Sam Houston State University* *Huntsville, TX 77341* *Phone: (936) 294-4319* *E-mail: mjs079@shsu.edu*	Sharla Miles Office of Research and Sponsored Programs Sam Houston State University Huntsville, TX 77341 Phone: (936) 294-4875 Email: irb@shsu.edu
Christina Smith Dept. of Forensic Science Sam Houston State University Huntsville, TX 77341 Phone: (936) 294-4319 E-mail: crs040@shsu.edu	*Dr. Madeleine Swortwood* Dept. of Forensic Science Sam Houston State University Huntsville, TX 77341 Phone: (936) 294-4319 E-mail: mjs079@shsu.edu	Sharla Miles Office of Research and Sponsored Programs Sam Houston State University Huntsville, TX 77341 Phone: (936) 294-4875 Email: irb@shsu.edu

☐ I understand the above and consent to participate.

☐ I do not wish to participate in the current study.

APPENDIX C

Oral Fluid Survey – ADHD Medication

1. Have you ever taken one of the following medications? *Circle all that apply*

   Adderall

   Vyvanse

   Ritalin

   Concerta

   Dexedrine

   Other: please list _____

2. When is the last time you took one of these medications? How much did you take?

   Name of medication: _____

   Time of last dosage: _____

   Amount taken: _____

3. How often do you take these medications? *(i.e.: daily, weekly, for midterms/finals)*
4. Are you prescribed one of these mediations by a physician? *Circle one: yes or no*

   If yes, at what dosage?

This information is confidential and anonymous.

The information on this survey cannot be used to identify you.

VITA

EDUCATION

PhD Sam Houston State University, *Huntsville, TX* 2016 - Present
Doctorate of Philosophy in Forensic Science
Graduation: December 2021

BS Texas A&M University, *College Station, TX* 2013 - 2016
Bachelor's of Science in Biology, Minor: Sociology
GPA: 3.6

RESEARCH EXPERIENCE

Graduate Research 2016 - Present
Sam Houston State University; (Advisor: Dr. Madeleine Swortwood)

- Development and validation of novel methods for novel psychoactive substances and cognitive stimulant drugs following established toxicological standards in various matrices using liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ-MS)
- Development of quantitative method in blood using supercritical fluid chromatography-triple quadrupole mass spectrometry (SFC-MS/MS)
- Development and optimization of isolation procedures using solid-phase extraction (SPE)
- Applied validated methods to authentic case samples: rat plasma, postmortem blood, oral fluid
- Perform maintenance operations and repairs on LC-QQQ-MS

Capstone Research Title: Quantification of U-47700 and its Metabolites in Plasma LC-MS/MS

Dissertation Title: Development and Validation of Toxicological Methods for Cognitive Stimulants in Traditional and Alternative Matrices

EMPLOYMENT HISTORY

Graduate Research Assistant September 2018 - Present
Sam Houston State University; Huntsville, TX

- Teaching Assistant for graduate level Forensic Instrumental Analysis lab – preparation of drug standards and laboratory supplies, assisting in conduction of experiments, data analysis
- Teaching Assistant for graduate level Forensic Toxicology lab – preparation of drug standards and laboratory supplies, assisting in conduction of experiments, data analysis
- Maintain laboratory cleanliness and follow procedures and protocols
- Aid the Department of Forensic Science with administrative tasks
- Lab maintenance and organization

Graduate Mentor – McNair Scholars Program September 2018 – August 2021
Sam Houston State University, Huntsville, TX

- Facilitate personal meetings with 25-30 scholars to ensure student academic and research success
- Prepare and conduct weekly workshops on graduate school preparation, admissions, graduate research, public speaking, and oral presentation skills

Student Intern Summer 2017
Drug Enforcement Administration (DEA) – South Central Laboratory; Dallas, TX

- Observation of drug chemists during case work.
- Work with evidence technicians and fingerprint analysis during daily operations.
- Monthly instrument maintenance.
- Independent fingerprint cross-over chemistry (heroin) research project.

Graduate Assistant – Impaired Driving Initiatives April 2017 – June 2018
Sam Houston State University; Huntsville, TX

- Assisting with preparation and follow up of IDI courses by organizing class rosters, registration forms, training materials, class exams, and DRE cards.
- These classes include: Advanced Roadside Impaired Driving Enforcement (ARIDE) and Drug Recognition Expert (DRE) certification and recertification courses
- Maintaining program database – both in office and online.

Student Intern – Criminal Investigation Division January 2016 – May 2016
College Station Police Department; College station, TX

- One semester internship in the Criminal Investigation Division.
- Assisted detectives and sergeants with audio and/or video recordings, evidence handling and documentation, file maintenance, and suspect or victim interviews.

Peer Mentor – Transfer Student Program May 2015 – May 2016
Texas A&M University; College Station, TX

- Worked as an academic and social mentor to incoming transfer students at Texas A&M University.
- Provided these students with resources or connected them to appropriate help when they encountered any problems in their first semester.
- Hosted events to keep them involved and held office hours for personal meetings.

RELEVANT COURSEWORK

- Forensic Toxicology (Lecture/Lab), Advanced Instrumental Analysis (Lecture/Lab), Forensic Instrumental Analysis (Lecture/Lab), Fundamentals of Research Methods, Controlled Substance Analysis, Advanced Mass Spectrometry, Graduate Forensic Statistics, Scientific Communications, Quality Assurance & Ethics, Separation Sciences, Forensic Seminar, Laboratory Management

PRESENTATIONS AND PUBLICATIONS

Publications:

Smith C.R., Swortwood M.J. Short- and long-term stability of methylphenidate and its metabolites in blood. *Journal of Analytical Toxicology*. 2021; 45, 863-869,
Truver M.T., Smith C.R., Garibay N., Kopajtic T.A., Swortwood M.J., and Baumann M.H. Pharmacodynamics and pharmacokinetics of the novel synthetic opioid, U-47700, in male rats. *Neuropharmacology*. 2020; 177.

Smith C.R., Truver M.T., Swortwood M.J. (2019) Quantification of U-47700 and its metabolites in plasma by LC-MS/MS. *Journal of Chromatography B.* 2019; 1112, 41-47.

Conference Presentations:

Smith C.R.*, Swortwood M.J. Long- and short-term stability of methylphenidate and its metabolites in blood. POSTER PRESENTATION. Society of Forensic Toxicologists Annual Meeting. Nashville, TN. 2021.

Li S.Y.*, Smith C.R., Bartock S.H., McClure F.L., Edinboro L.E., Swortwood M.J. Evaluation of alcohol markers in urine and oral fluid after Kombucha consumption. POSTER PRESENTATION. Society of Forensic Toxicologists Annual Meeting. Nashville, TN. 2021.

Smith C.R.*, Young M., Swortwood M.J. Chiral separation and analysis of methylphenidate, ethylphenidate and ritalinic acid in blood by LC-MS/MS. ORAL PRESENTATION. American Academy of Forensic Sciences Annual Meeting. Virtual. 2021.

Smith C.R.*, Vikingsson S., Kronstrand R., Swortwood M.J. Chiral separation and quantitation of methylphenidate, ethylphenidate and ritalinic acid in blood using supercritical fluid chromatography. ORAL PRESENTATION. Society of Forensic Toxicologists Annual Meeting. Virtual. 2020.

Li S.Y.*, Smith C.R., Bartock S.H., McClure F.L., Edinboro L.E., Swortwood M.J. Evaluation of alcohol markers in urine and oral fluid after Kombucha consumption. ORAL PRESENTATION. Society of Forensic Toxicologists Annual Meeting, Virtual. 2020.

Truver M.T.*, Smith C.R., Garibay N., Kopajtic T.A., Swortwood M.J., and Baumann M.H. (2020) Pharmacodynamics and pharmacokinetics of the novel synthetic opioid, U-47700, in male rats. POSTER PRESENTATION. Society of Forensic Toxicologists Annual Meeting. Virtual. 2020.

Smith C.R.*, Swortwood M.J. Chiral separation of methylphenidate, ethylphenidate, and ritalinic acid in blood. POSTER PRESENTATION. American Academy of Forensic Sciences Annual Meeting. Anaheim, CA. 2020.

Smith C.R.*, Truver, M.T., Swortwood M.J. Quantification of U-47700 and its metabolites in plasma by LC-MS/MS. ORAL PRESENTATION. Society of Forensic Toxicologists Annual Meeting. Minneapolis, MN. 2018.

HONORS AND AWARDS

Guadalupe V. and Daniel P. King Criminal Justice Endowed Scholarship Recipient, Sam Houston State University *(Fall 2020 – Spring 2021)*

FSF Emerging Forensic Scientist Award (Finalist), The American Academy of Forensic Science *(Spring 2020, Spring 2021)*

FSF Student Scholarship Award Recipient, The American Academy of Forensic Science *(Spring 2020, Spring 2021)*

ORSP Individual Scholarship Program Grant Recipient, Sam Houston State University *(Fall 2019)*

ROAD to PhD Scholarship, Sam Houston State University *(Fall 2018 – Fall 2020)*

Forensic Science Scholarship, Sam Houston State University *(Fall 2018 – Present)*

SKILLS

Instrumentation

- LC-MS/MS (Agilent Technologies)
- GC-MS (Agilent Technologies)

Software

- Agilent MassHunter (Qualitative and Quantitative Analysis)
- Agilent Optimizer
- Agilent ChemStation
- Microsoft Office Products
- GraphPad Prism

Forensic Evidence

- Collection, handling, processing

PROFESSIONAL AFFILIATIONS

Society of Forensic Toxicologists, Student Affiliate

American Academy of Forensic Sciences, Student Affiliate

ROAD to PhD, Scholar, Sam Houston State University

Society of Forensic Science, Vice President, Sam Houston State University

National Society of College Scholars, Member

Alpha Kappa Delta Honor Society, Member

PROFESSIONAL DEVELOPMENT

- Graduate and Undergraduate Instructional Academy (GUIA) Teaching Conference: Huntsville, TX (2019)
    - Courses included: Managing Conflict, FERPA, Student Centered Learning, Title IX, Classroom Management, Grading and Giving Criticism, Diversity in the Classroom
- DUID Oral Fluid workshop: Dallas, TX (2019)
- Social and Behavioral Research Students. CITI Program (2018)
- SOP Writing for ISO 17025 Accreditation, RTI International (2018)
- RTI Module- "Introduction to Uncertainty in Forensic Chemistry and Toxicology"
- Safety Training: OSHA laboratory training (2016-2021)
- Bloodborne Pathogen: Exposure in the Workplace (2016-2021)