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Transdermal and Oral dl-Methylphenidate-Ethanol Interactions in C57BL/6J Mice

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Transdermal and oral *dl*-methylphenidate – ethanol interactions in C57BL/6J mice

By

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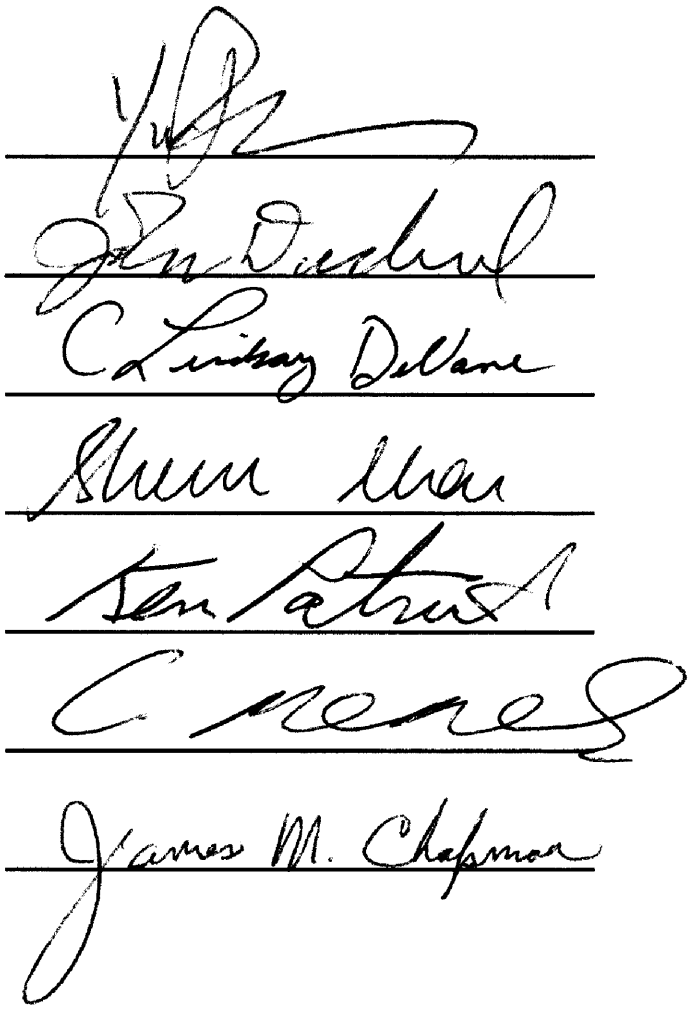

Acknowledgements

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To my friends and family who have always had faith in me even when I was certain that I was destined for failure, thank you.

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ABSTRACT

The persistence of attention-deficit/hyperactivity disorder (ADHD) into adulthood has been increasingly recognized over the past few decades and the stimulant drug *dl*-methylphenidate (MPH) has remained a first-line pharmacotherapeutic agent in the treatment of ADHD. Many adult ADHD patients who are prescribed MPH report concomitant use with ethanol.

In humans, coadministration of *dl*-MPH and ethanol results in pharmacokinetic and pharmacodynamic drug – drug interactions. Ethanol elevates biological concentrations of the pharmacologically active *d*-MPH isomer and yields the metabolic transesterification product ethylphenidate (EPH). EPH appears to be formed through the actions of carboxylesterase 1 (CES1) which exhibits *l*-MPH substrate enantioselectivity in both the metabolic transesterification and deesterification pathways. Accordingly, the mean absolute oral bioavailability of *l*-MPH is limited to only 1-3% compared to approximately 30% for *d*-MPH. However, dosing with transdermal *dl*-MPH (Daytrana®) avoids the extensive oral presystemic metabolism and leads to approximately 50 times more *l*-MPH reaching the systemic circulation when compared with oral dosing. Studies using human subjects are limited in their ability to examine abuse like doses.

Using a C57BL/6J mouse model, the experiments in this dissertation were designed to: 1) Establish the rewarding properties and abuse potential of i.v. *dl*-MPH as evidenced by drug seeking behavior; 2) Investigate the pharmacokinetic interactions of *dl*-MPH and ethanol coabuse, placing an emphasis on the MPH transdermal system; 3) Investigate the pharmacodynamic interactions of *dl*-MPH and ethanol coabuse.

The reward value of methylphenidate is evidenced by robust drug-seeking behavior in C57 mice, which are an appropriate model to investigate methylphenidate abuse liability. Pharmacokinetic studies showed that, as in humans, transdermal *dl*-MPH greatly facilitated the absorption of *l*-MPH in this mouse strain. Similarly, ethanol led to the enantioselective formation of *l*-EPH and to an elevation in *d*-MPH concentrations with both transdermal and oral *dl*-MPH. While only guarded comparisons between transdermal and oral *dl*-MPH can be made due to route-dependent drug absorption rate differences, transdermal *dl*-MPH was associated with significant MPH – ethanol interactions. Pharmacodynamic studies showed that an otherwise depressive dose of ethanol significantly potentiated oral *dl*-MPH induced increases in total distance traveled for the first 100 min. Further, transdermal *dl*-MPH increased total distance traveled after a latency of 80 min, though this effect was not potentiated by concomitant ethanol.

The results from these studies in combination with human data, provide a scientific basis for extending abuse precautions for the ethanol – *dl*-MPH combination in general, with a novel focus on transdermal *dl*-MPH.

Chapter 1

Introduction

Attention-Deficit/Hyperactivity Disorder: Prevalence & Etiology

Attention-deficit/hyperactivity disorder (ADHD) is the most commonly diagnosed childhood neuropsychiatric condition and the persistence of ADHD into adulthood is increasingly recognized [1-6]. Symptoms include inability to focus or pay attention, hyperactivity, and impulsive behaviors. There are 3 subtypes of ADHD: 1) Predominantly hyperactive-impulsive, 2) Predominately inattentive, 3) Combined hyperactive-impulse and inattentive [7]. In the United States, the lifetime prevalence for children and adolescents has been estimated to range upward to 9.0% [8]; and for adults the incidence appears to exceed 4% [9]. Boys are significantly more likely to be diagnosed with ADHD than girls [10], however recent studies suggest that this phenomenon could be in part due to subjectivity in referrals made by teachers [11].

There is no single cause for ADHD, however a number of factors can contribute to or worsen ADHD symptoms. These factors include: a genetic predisposition where prominent candidate genes include those expressing the D4 postsynaptic dopamine receptor and/or the presynaptic dopamine transporter (DAT), poor diet, neurochemical imbalances, e.g., dopamine and/or norepinephrine, and the social/physical environment.

Treatments

Following a diagnosis of ADHD, most patients undergo a combination of behavioral modifications and pharmacotherapy. There are two non-stimulant medications that are FDA approved in the treatment of ADHD,

atomoxetine (Strattera®) and guanfacine (Intuniv®). Atomoxetine is a selective norepinephrine reuptake inhibitor that is not a controlled substance, however disadvantages include rare, but severe, liver damage and suicide ideation [12]. Further, this drug is primarily metabolized by CYP2D6 whereby pharmacogenetic polymorphisms may become clinically significant in dose individualization. Guanfacine is an alpha-2 agonist and its mechanism of action appears to be through feedback inhibition of norepinephrine synaptic release. Only the extended-release formulation is FDA approved in the treatment of ADHD and the potential for cardiovascular side effects should be considered prior to use [13].

More commonly, ADHD patients are prescribed stimulant medications, e.g. methylphenidate (MPH) or amphetamine. Amphetamine is typically viewed as a 2nd line agent to treat ADHD not responsive to MPH. There are numerous amphetamine formulations used in the treatment of ADHD. These include "mixed amphetamine salts" composed of dl-amphetamine in an unusual 75% d- : 25% l-mixture of isomer; a prodrug derivative where d-amphetamine is converted to a lysine amide for reduced abuse potential and reported improvement in pharmacokinetic properties; and finally formulations containing only the more active d-isomer of amphetamine. As with most stimulant medications, abuse, dependence, and cardiovascular adverse events are major clinical consideration in the use of ADHD stimulants [14].

The racemic (50:50 mixture of enantiomers) stimulant drug *dl*-MPH (Figure 1.1) has remained the first-line pharmacotherapeutic agent to treat ADHD since the 1950s [3, 15-16]. MPH is generally an effective and well tolerated drug in the treatment of ADHD.

In the adult ADHD population, *dl*-MPH is also the most widely prescribed psychotherapeutic agent [5]. As a consequence, this controlled substance has become more widely available for abuse and diversion [17-19], especially among high school [20] and college students [21-22]. Appropriate drug therapy for an older ADHD population requires a special consideration of lifestyle and lifespan comorbidities [5], such as hypertension [21, 23], where elevation of blood pressure by *dl*-MPH can represent a contraindication.

d-MPH *l*-MPH

Figure 1.1 Enantiomers of MPH

Methylphenidate Pharmacokinetics

MPH is available both as an immediate-release tablet as well as in various modified-release formulations. The drug is subject to extensive and enantioselective presystemic metabolism. In humans, oral *dl*-MPH dosing results in only ~30% of the *d*-isomer and ~1% of the *l*-isomer reaching the systemic circulation. Mass balance studies conducted in humans and rodents demonstrate that ~90+% of the drug is hydrolyzed to the inactive [24] metabolite ritalinic acid [25], ~1-2% is p-hydroxylated, ~5% is oxidized to the corresponding lactam, and ~1-2% is excreted unchanged. MPH is not subject to metabolic isomerization. MPH exhibits the relatively short (2-3 h) half-life of 2-3 h largely due to the rapid hydrolysis of the methyl ester. Accordingly, most ADHD patients receiving immediate-release tablets require multiple daily doses to maintain symptom control[15]. This creates many issues related to convenience, compliance, peer ridicule and security of this schedule II narcotic, i.e., diversion. For these reasons, many MPH formulations incorporate a range of modified-release pharmaceutical technologies to allow for single daily dosing.

In 2006, the FDA approved the first transdermal patch for the administration of *dl*-MPH (Daytrana®). Like the modified-release oral formulations, the transdermal patch overcomes the need for multiple daily

dosing regimens, in this case by delivering in continuous release of *dl*-MPH throughout the 9 h recommended wear time (Figure 1.2).

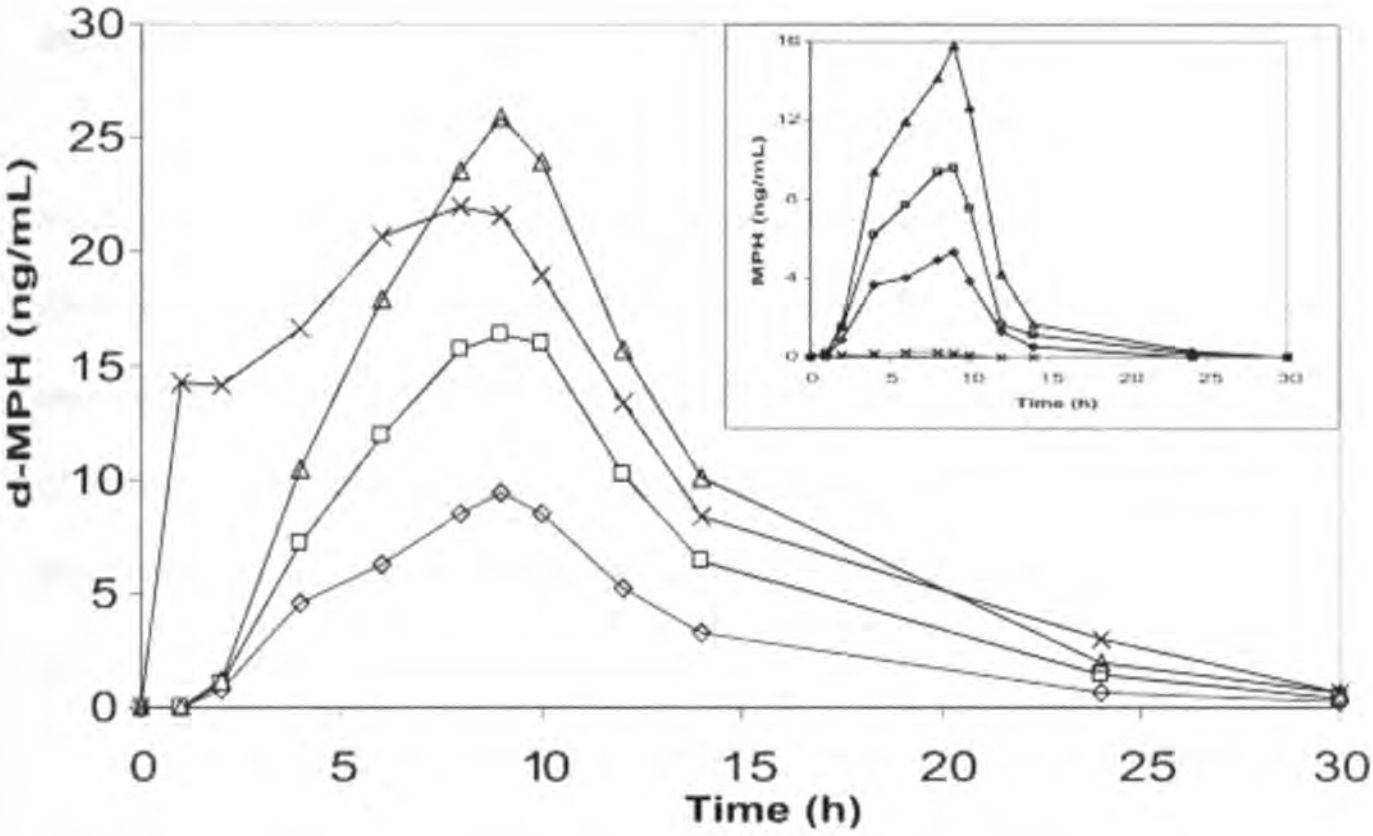


Figure 1.2 Plasma *d*-MPH and *l*-MPH (inset) concentration profiles for 12.5 (◊), 25 (□), and 37.5 cm^2 (Δ) transdermal methylphenidate compared to 54 mg Concerta® (X) [26].

This *dl*-MPH transdermal delivery system relies on a high load of *dl*-MPH free base incorporated within a uniform blend of acrylic polymers and silicone adhesives to drive drug absorption based on the drug concentration gradient, without the need for permeability enhancers (for review see [16]). Using transdermal delivery of *dl*-MPH circumvents the extensive and highly enantioselective presystemic metabolism associated with oral dosing [27-28].

This results in transdermal *dl*-MPH producing approximately 50 times higher plasma *l*-MPH concentrations than occurs following oral dosing (Figure 1.2) [29].

Methylphenidate: Interactions with Ethanol

Optimized adult ADHD pharmacotherapy may be complicated by alcohol consumption, alcohol use disorder (AUD) or other substance use disorders (SUD). SUD are over-represented in adult ADHD [1, 30-31]. The rewarding properties of *dl*-MPH have not been fully characterized and in light of the significant over-representation of SUD, e.g., AUD in adult ADHD, first line therapies such as *dl*-MPH require investigation of their abuse potential in the context of ethanol use and misuse.

In drug diversion, *dl*-MPH is reported to be co-abused with ethanol in the majority of users surveyed [18]. Not surprisingly, *dl*-MPH related emergency department visits number in the thousand each year [19]. Accordingly, prescribing *dl*-MPH has generated special concern regarding concomitant ethanol use or abuse [32-34]. This concern stems from the co-abuse of cocaine and ethanol as a precedent. Cocaine and methylphenidate are similar in their pharmacokinetic and pharmacodynamic profiles, where both increase extracellular dopamine through blockade of the DAT as well as increasing subjective reports of feeling "high" [18]. Both drugs contain a methyl ester metabolized by carboxylesterase 1 yielding a carboxylic acid

metabolite and, upon coadministration of ethanol, a transesterification metabolite [35].

Thus, coadministration of ethanol and *dl*-MPH orally to humans [27, 36] results in a drug – drug interaction where the methyl ester of MPH is transesterified to yield ethylphenidate (EPH; Figure 1.2) [27]. Both EPH and ritalinic acid formation appear to be primarily mediated by the actions of carboxylesterase 1 (CES1) [37-39] which exhibit *l*-MPH substrate enantioselectivity in both the transesterification and hydrolysis pathways [27, 40].

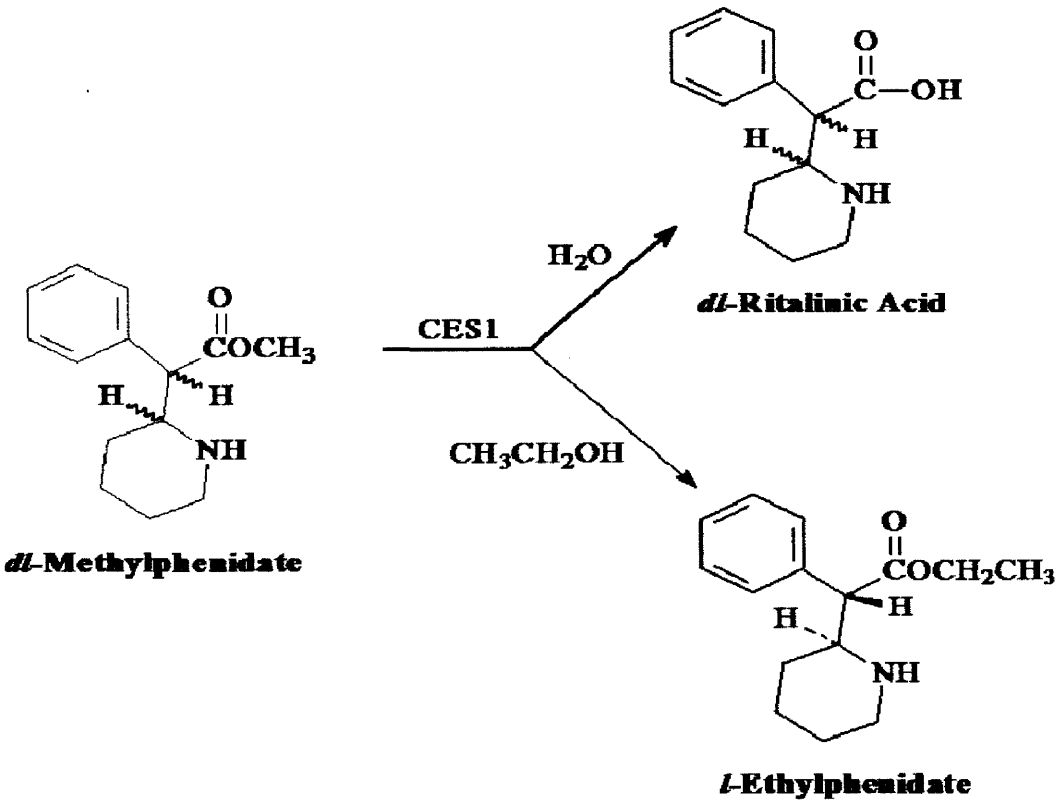


Figure 1.2 Metabolic fate of *dl*-MPH with or without ethanol.

Any *l*-MPH, or the metabolite *l*-EPH, which reaches system circulation is unlikely to contribute directly to the pharmacodynamics of the *dl*-MPH – ethanol interaction in view of the findings that only the *d*-isomers of MPH and EPH possess potent effects on dopaminergic and noradrenergic systems [40-41].

In a recent human study, coadministration of *dl*-MPH and ethanol resulted in a significant elevation of maximum plasma *d*-MPH concentrations (C_{max}) and overall *d*-MPH exposure [27]. Elevated plasma *d*-MPH concentrations increase the potential for adverse cardiovascular events [42-43] due to the fact that the *d*-isomer is responsible for adrenergic pressor effects. In addition to the influence of ethanol on *dl*-MPH pharmacokinetics, the above normal subjects reported an increase in pleasurable effects when combining *dl*-MPH with ethanol [44]. Such positive subjective effects may predispose individuals to greater abuse liability [32-33, 45]. The enhanced reward value of this drug combination may be based on interactive effects of these two psychoactive drugs on excitatory neural systems as recently been reported using a C57 mouse behavioral model [46]. Further, these increased effects may also pertain to the elevated *rate* at which *d*-MPH reaches the bloodstream, a temporal aspect associated with abuse potential [47-49]. Thus, when *dl*-MPH is combined with ethanol, the time to maximum concentration (T_{max}) occurs at the same time as *dl*-MPH dosed alone, however, the C_{max} has been found to be significantly higher at this time [27].

Societal/medical Implications of MPH-ethanol Interactions

There is an increasing number of adults being diagnosed with ADHD and most adult ADHD patients report ethanol use. Previous studies have shown a significant pharmacokinetic interaction between *dl*-MPH and ethanol in humans given therapeutic doses of oral *dl*-MPH [16, 27]. However, little work has addressed pharmacokinetic or pharmacodynamic interactions that may follow after abuse level dosing- bingeing. Further, the introduction of transdermal *dl*-MPH is of special concern due to the high levels of circulation *l*-MPH which are thought to enantioselectively interact and inhibit CES1. The interaction with ethanol could significantly alter the therapeutic effects of *dl*-MPH or contribute to side effects.

Therefore, the experiments described in this dissertation were designed to specifically address the following:

Specific Aim 1. Establish the rewarding properties of *dl*-MPH as evidenced by drug seeking behavior in a C57 mouse model.

1A. Determine if C57 mice will self-administer *dl*-MPH.

1B. Examine the drug seeking behavior of C57 mice for *dl*-MPH following increasingly difficult behavioral demands.

1C. Determine the maintenance of drug seeking behavior following a two week abstinence of any drug or cue.

Specific Aim 2. Investigate the effects of ethanol on the concentration of *d*-MPH and *l*- MPH in the blood, brain and urine of C57 mice.

2A. Develop new enantiospecific analytical methodology to establish the dose delivered by transdermal dosing for oral dose selection.

2B. Analyze brain, blood and urine concentrations of *d*-MPH and *l*-MPH following oral and transdermal dosing applying novel enantiospecific methodologies.

2C. Analyze micro-samples of brain, blood and urine for concentrations of *d*-MPH and *l*- MPH following an oral *dl*-MPH dose that reflects a comparable transdermal dose.

Specific Aim 3. Investigate stimulant effects of *dl*-MPH with or without ethanol on the locomotor activity of C57 mice.

3A. Develop methodology for analyzing locomotor activity of C57 mice given *dl*-MPH transdermally compared to oral dosing.

3B. Establish the interactive effects of ethanol and transdermal *dl*-MPH on the locomotor activity of C57 mice.

3C. Examine the interactive effects of ethanol and oral *dl*-MPH on the locomotor activity of C57 mice.

The results from these animal investigations will be discussed with a translational focus regarding the rational individualization of drug selection for vulnerable adult ADHD patients, i.e., those that consume and/or abuse ethanol.

Chapter 2

Reward value of methylphenidate in C57BL/6J mice

Introduction

The stimulant MPH provides a first-line pharmacotherapy for ADHD in both children and adults. There have been few animal studies modeling the abuse potential of MPH in the context of its reward properties. The Spontaneously Hypertensive Rat has been used in efforts to model the ameliorating effects of MPH on ADHD-like behaviors. While this rat appears to exhibit drug seeking behavior [50], other lines of evidence reveal that this particular rat does not model the control of ADHD symptoms by MPH [51-52]. Other studies using the Sprague-Dawley rat show that they also exhibit drug seeking behavior in response to MPH [53] and the response of this strain to stimulants of abuse has been well characterized. The current study aims to characterize the reward value of MPH in a novel mouse model.

Self-administration is an operant conditioning approach that gauges the reward properties of a particular drug. Typically, the test drug is administered intravenously. It is a widely utilized and well validated model of drug addiction liability and abuse potential. Reinstatement of drug seeking behavior after an extended abstinence period indicates the extent of addiction liability/reward value of the drug evaluated. Accordingly, we used this approach in the present study to measure the reward value of MPH in a novel C57BL/6J (C57) mouse model. The C57 mouse strain was chosen based on its frequent use as a reference strain in preclinical psychopharmacology of stimulant agents; including MPH [40, 46, 54-55]. Further, C57 mice have

been shown to self-administer cocaine [56] as well as reinstate cocaine seeking behavior in response to conditioned stimuli [57]. Cocaine is similar to MPH in its pharmacodynamic [58] and pharmacokinetic [35] characteristics, therefore we hypothesize that C57 mice will self-administer MPH and will act as a useful animal model for ongoing studies of MPH drug abuse pharmacology.

Materials and Methods

Animals

Experimentally naïve C57 male mice (n=8, 6-8 weeks old, Jackson Laboratories) were individually housed in an AALAC accredited animal facility and were maintained on a 12 h light cycle (lights on at 0600h). Behavioral testing occurred during the dark phase of their circadian cycle. The mice had free access to water, and food was restricted to maintain bodyweights at 90% of *ad libitum* weight after the jugular catheterization surgery. All experimental protocols were approved by the Institutional Animal Care and Use Committee and were consistent with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

Catheter Surgery

Mice were anaesthetized using gaseous isofluorane. Chronic indwelling catheters were designed and constructed for insertion into the right jugular vein with a skull-mounted access port as previously reported [56, 59]. Catheters were flushed daily with 0.1 mL of antibiotic (Cefazolin) followed by

0.1 mL of heparin. Mice were given 2 days for postoperative recovery before experimentation. Catheter patency was tested by injecting 0.1 mL of thiopental sodium on the day before and the last day of self-administration testing. Mice that did not lose muscle tone within 2-3 sec were excluded from the experiment.

Self-administration

Self-administration training was conducted during 2 h sessions on consecutive days. The self-administration chambers (Med Associates, Inc., Georgia, VT, model ENV-307A) were enclosed in sound attenuating cabinets. Presentation of stimuli and data collection was controlled by MedPC software. Catheters were connected to liquid swivels via silastic tubing. The swivels were suspended above the operant conditioning chamber and were connected to infusion pumps. Two response levers were located 6.5 cm above the grid floor on the same wall of the chamber. A reinforced response on the active lever resulted in 1) termination of the red house light, 2) initiation of a 2 sec compound conditioned stimulus consisting of a tone (2,900 Hz, ENV-323A), a white LED stimuli light (ENV-321M) located directly above the active lever, and the infusion pump noise, and 3) infusion of 0.1 mg/kg of MPH. Mice were first trained to press a lever according to a fixed ratio (FR) 1 schedule of MPH reinforcement with 2 sec time-out period. During the sessions, responses on the inactive lever had no programmed consequences but were recorded. Active and inactive lever assignments were randomized.

Daily self-administration training sessions were continued until the mouse reached the acquisition criterion (i.e. ≥ 10 infusions self-administered per session on a minimum of 5 consecutive training days). After acquisition on an FR1 schedule, mice were trained on an FR2 schedule using the same criteria and consequences for lever pressing. Following acquisition on an FR2 schedule, mice were finally trained on a progressive ratio (PR) 2 schedule using the same criteria and consequences for lever pressing.

Abstinence

Mice were maintained in their home cages for 14 consecutive days with food and water *ad libitum.*

Reinstatement testing

The first reinstatement test was conducted on an FR1 schedule, the second was conducted on an FR2 schedule, and the third was conducted on a PR2 schedule. During the test sessions, mice were connected to the silastic tubing previous used to deliver drug and lever presses were recorded for 2 h on the previously active and inactive levers with the tone and light consequences, but without any drug delivery.

Cue-less training

Mice were placed in the operant chamber for 2 h sessions for 2 weeks and there were no programmed consequences for either lever.

Statistical Methods

Repeated measures analyses of variance (ANOVA) were used to analyze MPH intake and lever responses. Lever (active vs. inactive) and day (self-administration, reinstatement, cue-less) were included as repeated measured factors. Statistical analysis was conducted using SPSS 12.0 (SPSS I.; Chicago, Illinois, USA). Statistically significant interaction effects were further investigated using Tukey post-hoc tests.

Results

All test animals met training criteria on an FR1 schedule within 10 days of the initial testing session demonstrating that C57 mice will self-administer MPH and acquire self-administration quickly as shown by mean lever presses on the active lever being significantly greater than the inactive lever. Moreover, self-administration of MPH was maintained despite increasingly difficult behavioral demands (Figure 2.1).

Following a 2 week abstinence period, lever pressing significantly increased on both the active and inactive levers during reinstatement training compared to the final training session at an FR 2 schedule (Figure 2.2). Further, the ratio of reinstatement over training mean lever presses on the active lever was significantly greater showing an increase in reward value through drug seeing behavior.

Removing the light and tone cues attenuated mean lever presses on both the active and inactive levers, but did not completely eliminate drug

seeking behavior implying that the drug seeking behavior is not simply a response to conditioned cues (Figure 2.3). Drug seeking behavior was significantly decreased during a cue-less testing session compared to a reinstatement testing session on an FR2 schedule.

Discussion

Previous studies using the spontaneously hypertensive rat as a model for ADHD show that they do exhibit drug seeking behavior [50]. However, growing evidence suggests that this rat is quite limited as an appropriate model for ADHD, particularly in their response to first-line therapeutic agent MPH [51-52]. The C57 mouse is a widely used reference strain for drugs of abuse [40-41, 46, 55]. C57 mice have been shown to self-administer cocaine [56] and in the present study have been shown to quickly acquire drug seeking behavior of MPH. Further, self-administration was maintained despite increasingly difficult demands. The special reward value of MPH is revealed through the robust drug-seeking behavior recorded, despite a two week abstinence period and the lack of drug reinforcement. In addition, we have demonstrated the maintenance of drug seeking behavior despite removal of condition cues which implies that the drug seeking behavior in this study was not based on a conditioned response, but rather due to the reward value of MPH itself. Our findings add support for the use of C57 mice as an appropriate reference stain and species to characterize MPH neuropharmacology in translational research.

Figure Legends

Figure 2.1

Mean lever presses on the active lever were significantly greater than the inactive lever on days 5-20. (*all $p>0.001$*)

Figure 2.2

Mean lever presses on both the active and inactive levers were significantly increased (*$p<0.001$) during reinstatement testing following a two week abstinence period.

Figure 2.3

Mean lever responses on both the active and inactive levers significantly decreased when the conditioned stimuli were removed, but were not completely eliminated. (*$p>0.001$).

Figure 2.1

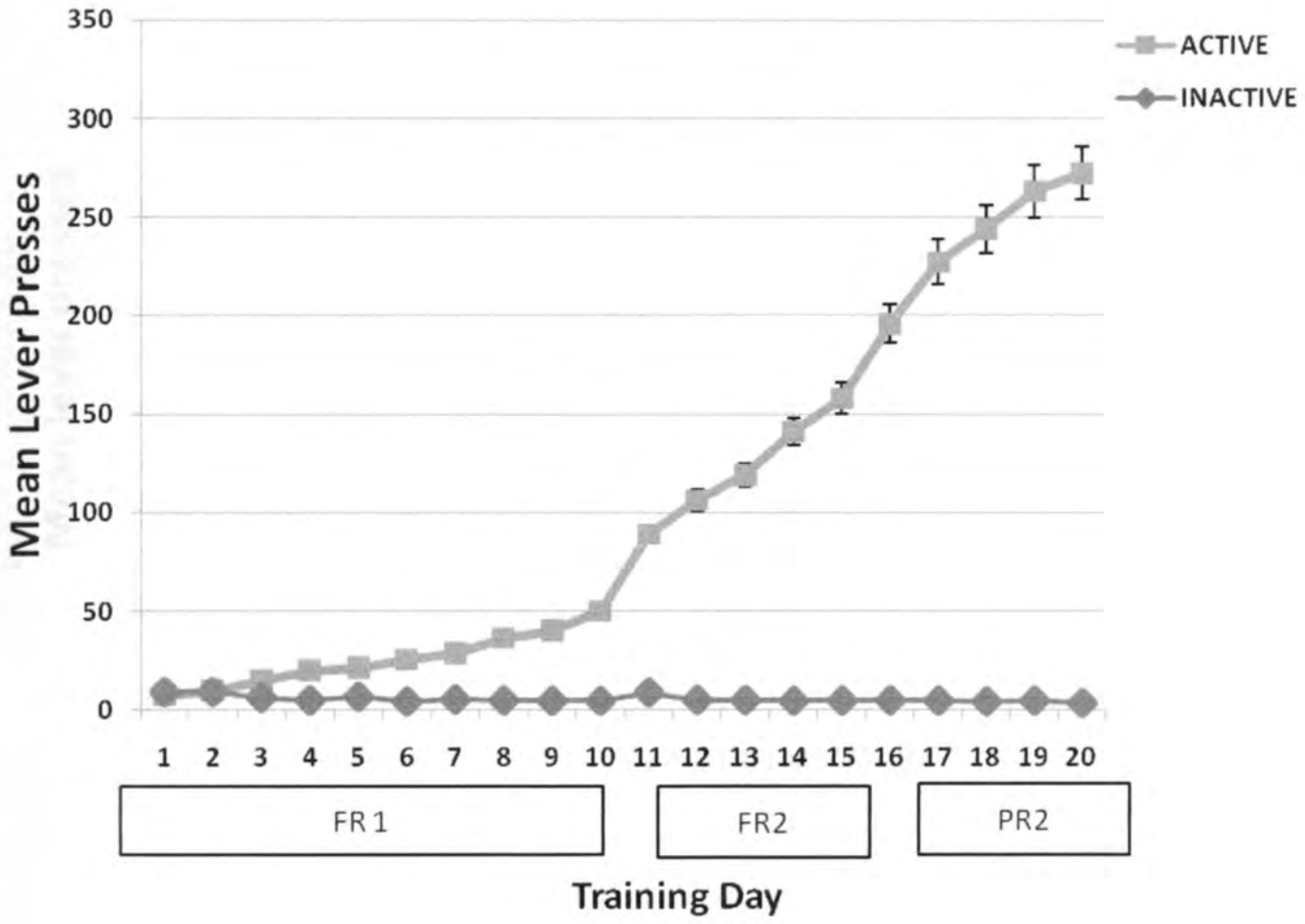
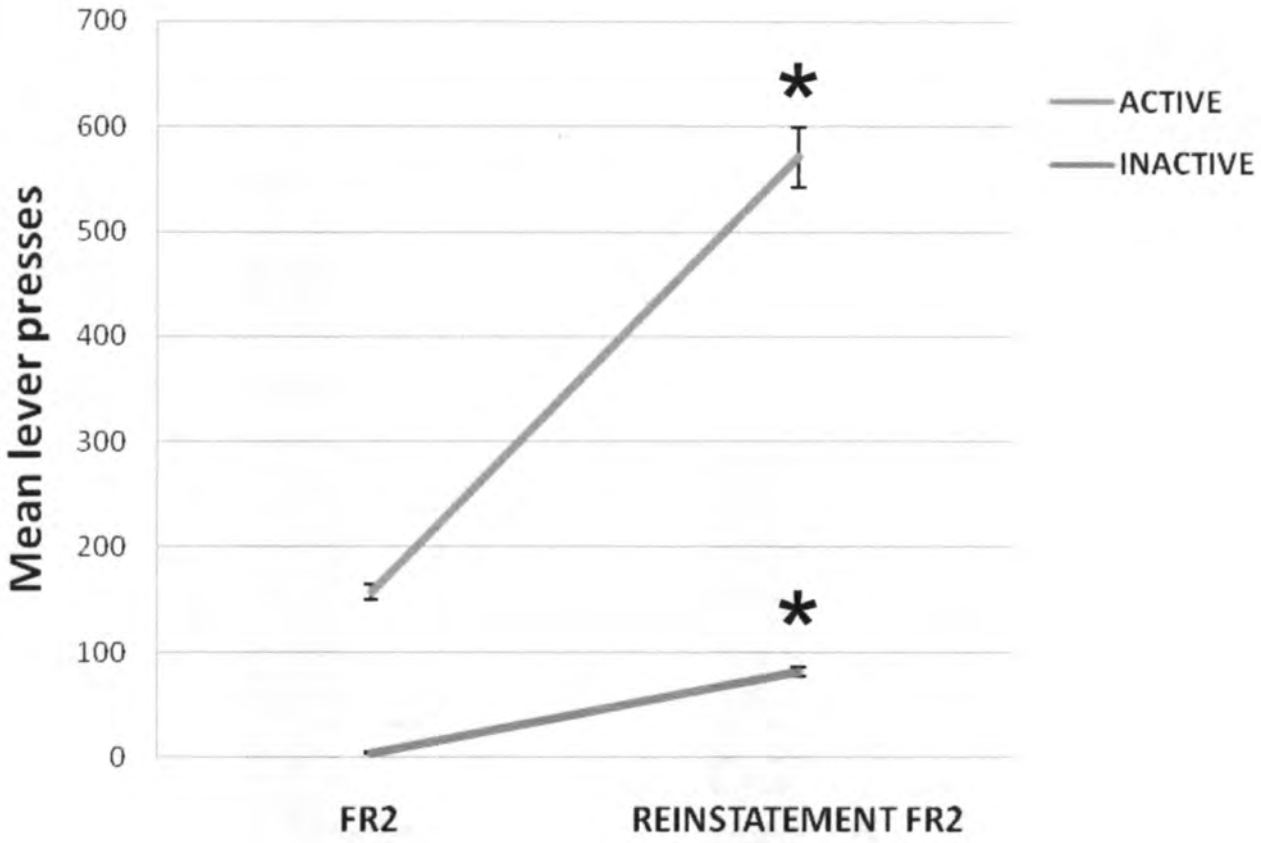

350
300
250
200
150
100
50
0
Mean Lever Presses
ACTIVE
INACTIVE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
FR 1
FR2
PR2
Training Day

Figure 2.2


700
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Mean lever presses
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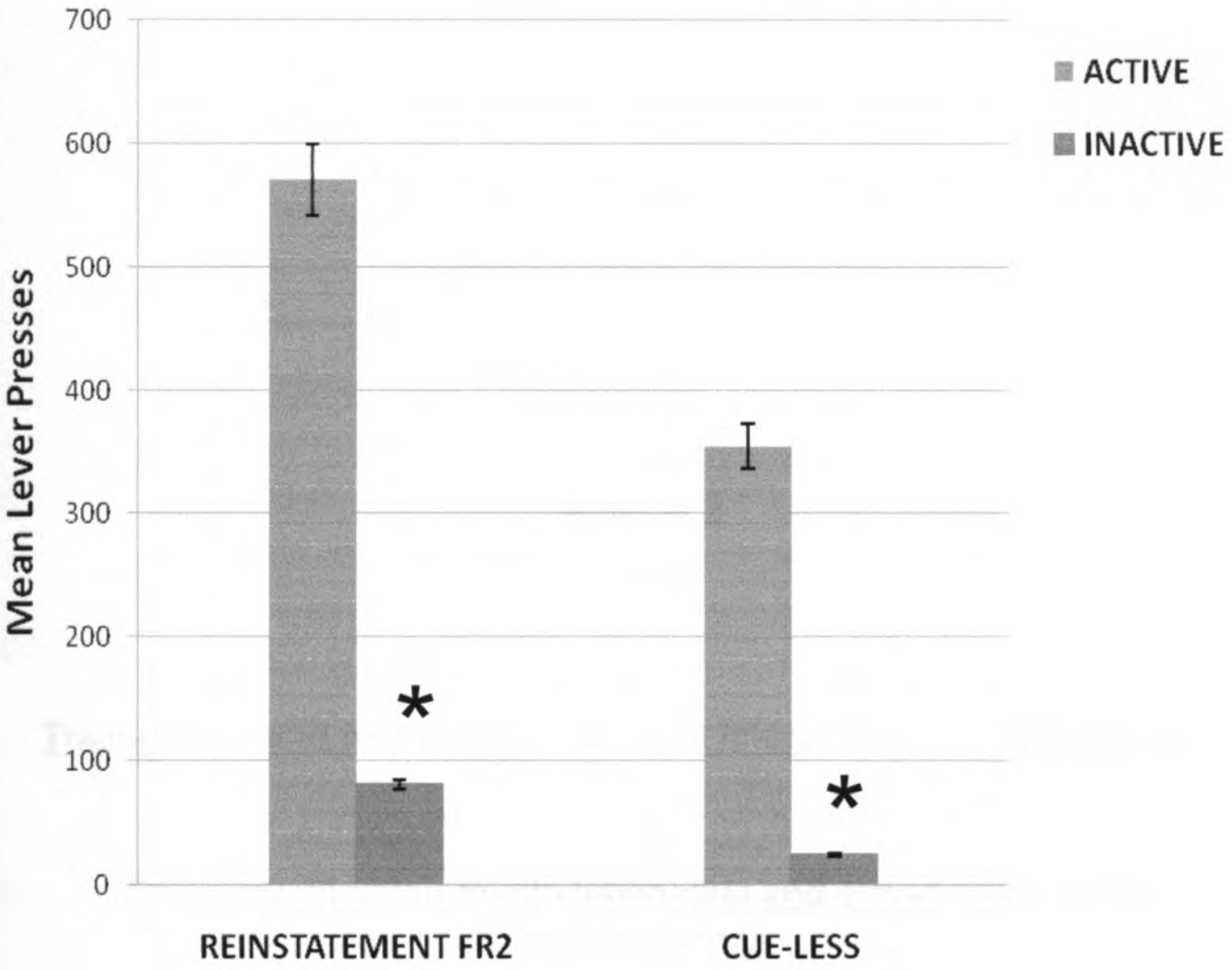
ACTIVE
INACTIVE
FR2
REINSTATEMENT FR2

Figure 2.3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REINSTATEMENT FR2
CUE-LESS

Chapter 3

Transdermal and oral *dl*-MPH – ethanol interactions in C57BL/6J mice:

Transesterification to ethylphenidate and elevation of *d*-MPH concentrations

Introduction

ADHD is the most commonly diagnosed childhood neuropsychiatric condition. The stimulant drug *dl*-MPH has remained a first-line pharmacotherapeutic agent to treat ADHD since the 1950s [3, 15-16]. Further, the persistence of ADHD into adulthood is increasingly recognized [1-6]. In the adult ADHD population, *dl*-MPH is also the most widely prescribed psychotherapeutic agent [5]. As a consequence, this controlled substance has become more widely available for abuse and diversion [17-19], especially among high school [20] and college students [21-22].

Appropriate drug therapy for this older ADHD population requires a special consideration of lifestyle and lifespan comorbidities [5], such as hypertension [21, 23]. Optimized adult ADHD pharmacotherapy may be complicated by alcohol consumption, alcohol use disorder (AUD) or other substance use disorders (SUD). Both AUD and SUD are over-represented in adult ADHD [1, 30-31], especially in women [60]. Not surprisingly, given the clinical nature of adult ADHD [61], and the susceptible population for which MPH is prescribed [1], *dl*-MPH related emergency department visits have numbered in the thousands each year, e.g., 8,000 for 2004 [19]. Moreover, emergency room presentations for incidents involving alcohol in combination with drugs have risen 63% for persons aged 18 to 19 years, and have increased 100% for persons age 45–54 [62]. Poison center data reveal how extensive *dl*-MPH abuse has become [19, 63-66]. In a drug diversion context,

ADHD stimulants are often co-abused with ethanol, e.g., in 53% of those surveyed [67]; and *dl*-MPH in particular has been reported to be co-abused with ethanol in 92% of those surveyed [18]. Accordingly, prescribing or diverting psychostimulants has generated special concern regarding concomitant ethanol use or abuse [32-34].

These statistics are consistent with MPH being classified as a DEA schedule II controlled substance [68], i.e., a medication of very high abuse potential [44, 69-70]. Accordingly, the prevalence and inherent danger of concomitant *dl*-MPH and ethanol warrant research into the pharmacology of this drug combination.

Coadministration of ethanol and *dl*-MPH orally to humans [27, 36] results in a drug – drug interaction where the methyl ester of MPH is transesterified to yield ethylphenidate (EPH; Figure 3.1) [27] in addition to being hydrolyzed to the inactive [24] metabolite ritalinic acid [25]. Both EPH and ritalinic acid formation appear to be primarily mediated by the actions of carboxylesterase 1 (CES1) [37-39] which exhibits *l*-MPH substrate enantioselectivity in both the transesterification and hydrolysis pathways [27, 40].

The metabolic transesterification of *dl*-MPH with ethanol to yield EPH was first reported in vitro using rat microsomes [71]. Subsequently, EPH was detected in human tissues from two fatal drug overdoses in which unknown amounts of MPH and ethanol were consumed [72]. These findings prompted

a normal human volunteer pilot study of the *dl*-MPH – ethanol interaction [73], followed by a larger human study where enantiospecific methodology for plasma analysis was utilized [44]. In this latter study, it was established that the *dl*-MPH – ethanol transesterification pathway primarily yields the *l*-enantiomer of EPH (Figure 3.1).

Any *l*-MPH, or the metabolite *l*-EPH, which reaches the bloodstream is unlikely to contribute directly to the pharmacodynamics of the *dl*-MPH – ethanol interaction in view of the findings that only the *d*-isomers of MPH and EPH possess potent effects on dopaminergic and noradrenergic systems [40-41]. This notwithstanding, ethanol consumed with *dl*-MPH by normal human volunteers resulted in a significant elevation of maximum plasma *d*-MPH concentrations (C_{max}) and overall *d*-MPH exposure [27]. Elevated plasma *d*-MPH concentrations increase the potential for adverse cardiovascular events, especially in ADHD patients with comorbid hypertension [42-43].

In addition to the influence of ethanol on *dl*-MPH pharmacokinetics, the above normal subjects reported an increase in pleasurable effects when combining *dl*-MPH with ethanol [44]. Such positive subjective effects may predispose individuals to greater abuse liability [32-33, 45]. The enhanced likability of this drug combination may be based on interactive effects of these two psychoactive drugs on excitatory neural systems as recently reported using a C57BL/J6 (C57) mouse behavioral model [46]. However, the increased likability may also pertain to the elevated rate at which *d*-MPH

reaches the bloodstream [47-49]. When *dl*-MPH is combined with ethanol, the time to maximum concentration (T_{max}) occurs at the same time as *dl*-MPH dosed alone. However, the C_{max} is much higher at this time following concomitant *dl*-MPH and ethanol than when *dl*-MPH is dosed alone [27].

In 2006, the FDA approved the first transdermal patch for the administration of *dl*-MPH (Daytrana®). This *dl*-MPH transdermal delivery system (MTS) relies on a high load of *dl*-MPH free base incorporated within a uniform blend of acrylic polymers and silicone adhesives to drive drug absorption based on the drug concentration gradient, without the need for permeability enhancers (for review see [16]). Using transdermal delivery of *dl*-MPH circumvents the extensive and highly enantioselective presystemic metabolism associated with oral dosing [27-28]. Accordingly, MTS results in approximately 50 times higher plasma *l*-MPH concentrations than occur following oral dosing [29].

The present preclinical study investigated aspects of MTS and oral MPH absorption and disposition as influenced by the coadministration of ethanol. Special attention was given to the formation of *l*-EPH in view of the relatively large amount of *l*-MPH anticipated to reach the bloodstream following MTS delivery. The C57 mouse strain was chosen based on its frequent use as a reference strain in preclinical psychopharmacology of stimulant agents; including MPH and ethanol [40-41, 46, 55, 74]. Further, like human MPH metabolism, the C57 mouse has previously been reported to

favor *l*-MPH as a substrate in the transesterification of ethanol to yield *l*-EPH after intraperitoneal (i.p.) dosing [40].

Blood, brain and urine concentrations of *d*-MPH, *l*-MPH, *d*-EPH and *l*-EPH were analyzed. The mean MTS dose delivered from a ¼ of a 12.5 cm^2 patch (smallest of 4 sizes available) after a 3.25 h wear was calculated by quantifying the residual MPH content in the used patches. This dose was then administered for oral studies, while clearly recognizing the limitations of any direct drug dispositional comparisons of a bolus oral *dl*-MPH dose to that of the MTS in mice where prolonged release of drug occurs from the patch. A modification of an established gas chromatographic-mass spectrometric-electron impact-selected ion monitoring (GC-MS-EI-SIM) method was used for these enantiospecific determinations [41, 75]. MPH and EPH enantiomers were derivatized with (*S*)-N-trifluoroacetylprolyl chloride (TFP-Cl) to yield GC resolvable diastereomers. Piperidine-deuterated *dl*-MPH was incorporated for analytical control.

Materials and Methods

Materials

Ethanol used for oral animal studies was from AAPER Alcohol and Chemical Co. (Shelbyville, KY; 95%). *dl*-MPH·HCl used for oral animal studies was from Sigma-Aldrich (St. Louis, MO; lot # 118K1052) and the 12.5 cm^2 size MTS was from Shire US (Wayne, PA; lot # 2616811; smallest of 4

sizes available). Laboratory tape used to secure MTS or placebo was from VWR International (white, 12.7 mm). *dl*-MPH·HCl in methanol (1 mg/mL calculated as free base; Cerilliant, Round Rock, TX) was used as the analytical reference standard. The *dl*-EPH·HCl standard in ethanol (1 mg/mL calculated as free base) was synthesized in-house[41]. (*S*)-N-(trifluoroacetyl)prolyl choride in dichloromethane (1M; Aldrich-Aldrich), sodium carbonate (Fischer Scientific, Fair Lawn, NJ), n-butyl chloride (Burdick & Jackson, Muskegon, MI) and acetonitrile (Mallinckrodt Inc, Paris, KT) were used. Piperidine deuterated *dl*-MPH·HCl was synthesized in-house[76] and contained approximately 25% of the D_5-isotopolog for SIM monitoring and containing no $D_{0\text{-}1}$-MPH. It is noted that piperidine deuterated D_9-MPH·HCl is commercially available (Cerilliant).

Animals

Male C57 mice aged 8-10 weeks (25-35 g) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). They were individually housed in a temperature and humidity controlled colony room on a 12-h light/dark cycle (light: 07.00–19.00 h) with free access to food and water for at least 7 days before the start of any tests. All experiments were approved by and conducted within the guidelines of the Institutional Animal Care and Use Committee at the Medical University of South Carolina and followed the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH

Publication no. 80–23, revised 1996). Animal studies were conducted in the Institute of Psychiatry.

Drug Administration

Mice were randomly placed into 1 of 4 test groups as shown in Table 1. All mice, regardless of group assignment, were treated similarly. This included the use of active (MTS) or placebo patches and delivery of ethanol or water by gastric intubation (gavage). To this end, mice were lightly anesthetized by placement into a chamber containing 5% isofluorane for 8-10 min. The mice were removed and their hair was clipped along their abdomen and back, from shoulders to hips.

Immediately after clipping hair, ¼ of a 12.5 cm^2 MPH transdermal patch, or a placebo patch (band-aid adhesive resembling the MTS), was applied to the lower left hip area. The patch was secured by applying tape over the patch and around the mouse for one full loop in order to ensure a constant skin interface and to prevent the mice from disturbing the patch. Mice were returned to their home cage for 15 min to recover from anesthesia, then dosed by gavage, according to their assigned group, i.e., 3.0 g/kg ethanol and 7.5 mg/kg (calculated as the free base) *dl*-MPH·HCl, or deionized water (dH_20) using a standard volume of 0.02 mL/g body weight.

Sample collection

Following gavage, mice were individually placed for 3 h in single metabolic chambers designed to separate urine from solid waste. Urine was collected and measured to the nearest µL. Mice were then deeply

anesthetized using isofluorane. Venous blood was collected using cardiac puncture and stored in heparinized tubes. The brain was removed, separated along the sagittal line, weighed, and stored as 2 separate samples. Used patches were collected and later extracted for residual *dl*-MPH to calculate the dose delivered to the cutaneous site. Blank urine, blood, and brain used for calibration curves were collected from mice not exposed to any drugs. All matrices were kept on dry ice until stored in a -70°C freezer.

Sample Preparation

Urine

All urine samples were thawed immediately prior to analysis. Blank mouse urine (150 µL) was fortified with *dl*-MPH over a range of concentrations (0, 0.5, 0.75, 1.5, 3, 4.5 µg/mL) and *dl*-EPH (0, 0.15, 0.3, 0.45, 0.6, 0.9 µg/mL) (Figure 3.4). These calibrators were run in parallel with experimental urine samples (150 µL). The internal standard, piperidine deuterated *dl*-MPH, was dissolved in dH_20 such that 200 µL aliquots provided a concentration of 5 µg D_5-*dl*-MPH/150 µL of urine. Sodium carbonate (50 µL; 1.2 M) was added to each urine sample to adjust the pH to approximately 9.5. Samples were extracted with n-butyl chloride: acetonitrile (2 mL; 4:1) by vortexing for approximately 0.5 min.

Blood

All blood samples were thawed immediately prior to analysis and used in the freezer- hemolyzed state in view of MPH having previously been

reported to distribute nearly equally between serum and the red cell fraction [77]. Blank mouse blood (200 µL) was fortified with *dl*-MPH over a range of concentrations (0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0 µg/mL) and with *dl*-EPH (0, 0.01, 0.025, 0.05, 0.075, 0.1 µg/mL). These were run in parallel with experimental blood (200 µL) as calibrators. The internal standard, piperidine deuterated *dl*-MPH, was dissolved in dH_20 such that 200 µL aliquots provided a concentration of 5 µg D_5-*dl*-MPH/200 µL of blood. Sodium carbonate (2mL; 1.2 M) was added to each blood sample to adjust the pH to approximately 9.5. Samples were extracted with n-butyl chloride: acetonitrile (2 mL; 4:1) by vortexing for approximately 0.5 min.

Brain

All brain samples were thawed immediately prior to analysis. Blank mouse brain (1/2, left hemisphere) was fortified with *dl*-MPH over a range of concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5 µg/g) and with *dl*-EPH (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 µg/g) and run in parallel with experimental brains (left hemisphere). The internal standard, piperidine deuterated *dl*-MPH, was dissolved in dH_20 such that 200 µL aliquots provided a concentration of 5 µg D_5-*dl*-MPH/150 µL of urine. The internal standard, piperidine deuterated *dl*-MPH, was dissolved in dH_20 such that 200 µL aliquots provided a concentration of 2.5 µg D_5-*dl*-MPH/ brain sample. Sodium carbonate (2mL; 1.2 M) was added to each brain sample to adjust the pH to approximately 9.5. Samples were homogenized (Polytron PT1200) for 10 sec, then 0.5 g sodium

chloride was added and the samples were vortexed for 20 sec. Samples were extracted with n-butyl chloride: acetonitrile (2 mL; 4:1) by vortexing for 30 sec, then centrifuged at 3000 rpm for 7 min.

MPH extraction from used patches

Used patches were analyzed for residual content of *dl*-MPH to establish the cutaneous dose delivered. Before being placed on the animal, whole patches (including the backing) were weighed and then cut into quarters. Each ¼ was then weighed and used to determine what percent of the whole patch it represented.

In advance of analyzing the used patches for their dl-MPH content, a method for *dl*-MPH recovery from unused patches was developed. The unused patches were placed in scintillation vials with methanol (1 mL/calculated mg of *dl*-MPH) and sonicated over a range of times from 1 min to more than 20 min to determine the time required for near complete extraction/recovery. An unused 12.5 cm^2 patch contains 27.5 mg of *dl*-MPH free base whereby a ¼ patch contains 6.875 mg of *dl*-MPH. For specific ¼ patch cuttings, the exact *dl*-MPH content was calculated as follows: (Weight of ¼ MTS / Weight of whole MTS) x 27.5 = mg *dl*-MPH. Accordingly, for the used study patches, residual *dl*-MPH was determined by taking a 100 µL aliquot after 15 min of sonication and adding D_5-*dl*-MPH (10 µg) as the internal standard.

Chiral derivatization

The organic phases from all matrix extractions were transferred into 4 mL screw-cap silanized vials (Supelco) and the solvent was evaporated to dryness under nitrogen. TFP-Cl (1M; 250 µL) was added to each vial, sealed with Teflon® lined caps (Supelco) and heated at 58 °C for 45 min. Aliquots of these samples were then transferred to silanized microvial inserts within auto sampler vials for GC-MS analysis.

Instrumental analysis

All analyses were conducted using an Agilent Model 6890 GC-5973N MS with ChemStation using a modification of published methods [41, 75]. GC separations were on a 30 m x 0.32 mm, 0.25 µm film thickness, 5% phenylmethylpolysiloxane fused-silica column (DB-5 J & W Scientific, Folsom, CA). Pulsed-splitless injections (2 µL) were used. The injector port was fit with a deactivated glass wool protected sleeve operated at 250 °C and the helium carrier gas linear velocity was 50 cm/s. The GC was held at 70° C for 1.5 min, then ramped to 315 °C at 10° C/min and held for 4 min for a total run time of 30 min. Detection was by EI ionization (70 eV) and SIM, acquiring the N-TFP-piperidyl fragment ions of *d*-MPH, *l*-MPH, *d*-EPH and *l*-EPH (*m/z* 277) with D_5-*d*-MPH and D_5-*l*-MPH monitored at *m/z* 282 (Figure 3.2).

The lower limit of quantitation was based on a signal-to-noise ratio of ≥10 for all analytes. The signal-to-noise ratios for the lowest calibrators were ≥ 25. It is noted that calibrator concentrations are indicated as racemic (*dl*-)

MPH and EPH, while analyte concentrations are reported for each enantiomer. All calibration plots provided linearity of $r^2 > 0.99$ (Figure 3.3).

Statistical Methods

A two way analysis of variance (ANOVA) followed by pair wise comparisons using the Student's t-test method was used in the analysis of all data. Samples were analyzed as independent samples and were assumed to have equal variances. Statistical analysis was conducted using SPSS 12.0 (SPSS I.; Chicago, Illinois, USA).

Results

MPH dose delivered from MTS

The *dl*-MPH dose received by the MTS test animals over the 3.25 h wear time was determined by extracting the remaining *dl*-MPH from used patches and back calculating from the initial *dl*-MPH content in a ¼ of a 12.5 cm^2 MTS (Figure 3.4). Sonication for 15 min was necessary to extract a mean no less than 95% of the labeled *dl*-MPH content of unused ¼ patches and, accordingly, 15 min of sonication was used to calculate the 3.25 h dose delivered by difference (Figure 3.5). Shorter sonication times did not allow for complete *dl*-MPH extraction, while using later time points caused the MTS matrix to significantly degrade. This resulted in the extractant becoming cloudy and GC-MS of such aliquots were found to foul the injector port and result in unacceptable chemical noise in the chromatograms. The mean *dl*-

MPH dose delivered using the MTS over 3.25 h was 0.23 mg or 7.5 mg/kg. This dose was used for oral dosing (gastric intubation) in a parallel study of oral *dl*-MPH – ethanol interactions. The 7.5 mg/kg oral dose is likely to over-represent the bioavailable fraction of the mean *dl*-MPH MTS dose calculated as above in view of the likelihood of some residual *dl*-MPH remaining in the skin prior to circulatory absorption, e.g., in humans dosed with MTS, residual *dl*-MPH results in a biphasic decay of the drug from plasma following patch removal [78].

Influence of ethanol on urinary analytes

Transdermal dl-MPH

The total urinary elimination of *d*-MPH following the 3.25 h MTS wear time was significantly greater in the animals dosed with ethanol compared to those given dH_20 (Figure 3.6a; $t = 5.52$, $df = 10$, $p<0.001$); rising from 0.48 µg to 1.39 µg to account for 0.04% of the total dose of *d*-MPH calculated to be cutaneously delivered. Further, in animals dosed with MTS, total urinary excretion of *l*-MPH was significantly increased, rising from 0.43 µg for animals dosed with dH_20 to 0.96 µg for animals dosed with ethanol (Figure 3.7a; $t = 4.07$, $df = 10$, $p<0.01$). There was not a significant difference between the urinary excretion of *d*-MPH compared to *l*-MPH in animals dosed with dH_20, however, in animals dosed with ethanol the urinary excretion of *d*-MPH was significantly greater than *l*-MPH ($t = 2.13$, $df = 10$, $p<0.05$). Both enantiomers of EPH were detectable in animals gavaged with ethanol, however, *l*-EPH

was enantioselectively formed with a significantly greater total elimination found relative to *d*-EPH (Figure 3.8a; t = 5.74, df = 10, p<0.001). The total urinary elimination of *l*-EPH was 0.2 µg which represents 0.01% of the total dose of *l*-MPH calculated to be cutaneously delivered, while the total urinary elimination of *d*-EPH was 0.05 µg. The total urine volume excreted following ethanol treatment was significantly greater than following dH_20 treatment (t = 4.81, df = 10, p<0.001) as consistent with the diuretic effect of ethanol.

Oral dl-MPH

The total urinary elimination of *d*-MPH following oral *dl*-MPH over the 3 h collection period was significantly greater in the animals dosed with ethanol compared to those given dH_20 (Figure 3.6b; t = 7.56, df=10, p<0.001); rising from 0.09 µg to 0.46 µg and accounting for 0.012% of the total dose of *d*-MPH gavaged. Further, in animals dosed with oral *dl*-MPH, the total urinary excretion of *l*-MPH was significantly increased, rising from 0.07µg for animals dosed with dH_20 to 0.31µg for animals dosed with ethanol (Figure 3.7b; t = 5.45, df= 10, p<0.001). There was not a significant difference between the urinary excretion of *d*-MPH compared to *l*-MPH in animals dosed with dH_20, however, in animals dosed with ethanol the urinary excretion of *d*-MPH was significantly greater than *l*-MPH (t = 2.23, df = 10, p<0.05). Both isomers of EPH were detectable in animals gavaged with ethanol, however, *l*-EPH was enantioselectively formed with a significantly greater total urinary elimination of *l*-EPH relative to *d*-EPH (Figure 3.8b; t = 3.71, df = 10, p<0.01). The total

urinary elimination of *l*-EPH was 0.02 µg, while the total urinary elimination of *d*-EPH was 0.005µg. Again, the total urine volume excreted followed ethanol (a diuretic) treatment was significantly greater than following dH_20 treatment ($t = 4.39$, $df = 10$, $p<0.001$).

Influence of ethanol on blood analytes

Transdermal dl-MPH

The blood concentration of *d*-MPH after MTS dosing was significantly greater in animals dosed with ethanol compared with dH_20; increasing 72% from 0.36 µg/mL to 0.61 µg/mL (Figure 3.6a; $t = 4.22$, $df = 10$, $p<0.01$),. Further, in animals dosed with MTS, concentrations of *l*-MPH significantly increased from 0.29 µg/mL for animals dosed with dH_20 to 0.51 µg/mL for animals dosed with ethanol (Figure 3.7a; $t = 2.82$, $df = 10$, $p<0.05$). There was no significant difference between the blood concentration of *d*-MPH and *l*-MPH in animals dosed with dH_20 or in animals dosed with ethanol. Both enantiomers of EPH were formed in animals gavaged with ethanol, however, *l*-EPH was enantioselectively formed with a significantly greater concentration found relative to *d*-EPH (Figure 3.8a; $t = 2.99$, $df = 10$, $p<0.05$). The blood concentration of *l*-EPH was 0.04 µg/mL, while the concentration of *d*-EPH was 0.03 µg/mL.

Oral dl-MPH

The blood concentration of *d*-MPH following oral *dl*-MPH was significantly greater in the animals dosed with ethanol compared to those

given dH_20; increasing 59% from 0.018 µg/mL to 0.03 µg/mL (Figure 3.6b; t = 2.95, df = 10, $p<0.05$). Further, in animals dosed with oral *dl*-MPH, concentrations of *l*-MPH were significantly increased from 0.015 µg/mL for animals dosed with dH_20 to 0.05 µg/mL for animals dosed with ethanol (Figure 3.7b; t = 4.56, df = 10, $p<0.001$). There were no significant differences between the blood concentration of *d*-MPH and *l*-MPH in animals dosed with dH_20 or in animals dosed with ethanol. Neither isomer of EPH was detectable in animals gavaged with oral *dl*-MPH and ethanol.

Effect of ethanol on brain analytes

Transdermal dl-MPH

The brain concentration of *d*-MPH after MTS dosing was significantly greater in animals dosed with ethanol compared the dH_20 group; increasing 65.3%from 0.81 µg/g to 1.34 µg/g (Figure 3.6a; t = 2.89, df =10, $p<0.05$). Further, in animals dosed with MTS, concentrations of *l*-MPH were significantly increased by ethanol, rising from 0.84 µg/g for animals dosed with dH_20 to 1.33 µg/g for animals dosed with ethanol (Figure 3.7a; t = 2.18, df =10, $p<0.05$). There were no significant differences between the brain concentration of *d*-MPH and *l*-MPH in animals dosed with dH_20, or in animals dosed with ethanol. Both isomers of EPH were formed in animals gavaged with ethanol, however, *l*-EPH was enantioselectively formed with a significantly greater concentration found relative to *d*-EPH (Figure 3.8a; t =

8.57, df = 10, $p<0.001$). The brain concentration of *l*-EPH was 0.14 µg/g, while that of *d*-EPH was 0.005 µg/g.

Oral dl-MPH

The brain concentration of *d*-MPH following oral dl-MPH was significantly greater in the animals dosed with ethanol compared to those given dH_20; increasing 40.6% from 0.03 µg/g to 0.05 µg/g (Figure 3.6b; t = 3.67, df = 10, $p<0.01$). Further, in animals dosed with oral *dl*-MPH, concentrations of *l*-MPH were significantly increased from 0.02 µg/g for animals dosed with dH_20 to 0.06 µg/g for animals dosed with ethanol (Figure 3.7b; t = 3.83, df = 10, $p<0.01$). There were no significant differences between the brain concentration of *d*-MPH and *l*-MPH in animals dosed with dH_20 or in animals dosed with ethanol. Both isomers of EPH were formed in animals gavaged with ethanol; however, *l*-EPH appeared to have been enantioselectively formed, though the mean concentration was not significantly different from that of *d*-EPH (Figure 3.8b).

Discussion

Oral *dl*-MPH in humans is subject to pronounced enantioselective first-pass metabolism which limits *l*-isomer systemic exposure to approximately 1% that of *d*-MPH [28]. The mean absolute bioavailability of *dl*-MPH has been reported to be 30%, but ranges from 11-51% [79-80]. In effect, first-pass metabolism biocatalytically "resolves" oral *dl*-MPH [81], resulting in only the *d*-

isomer appreciably reaching the bloodstream. The *d*-isomer component of *dl*-MPH is generally regarded as the pharmacologically active isomer, responsible for efficacy in the treatment of ADHD [82-83]. The low oral bioavailability of *dl*-MPH is largely due to the facile hydrolysis of the constituent methyl ester to yield the inactive [24] metabolite *dl*-ritalinic acid and catalyzed primarily through the actions of CES1 [37-39, 84]. This facile pathway limits the half-life of *dl*-MPH to only 2-3 h [85]. Approximately 1% of MPH is excreted in urine unchanged in humans over 24 h, and excreted predominantly as the *d*-isomer [75].

Our studies with mice dosed with oral *dl*-MPH (7.5 mg/kg) and dH_20, while being limited to a single 3 h time point for blood and brain sampling, suggest a lower degree of metabolic enantioselectivity relative to humans, whereby the *d*-MPH-to-*l*-MPH ratio for blood and brain were 1.22 and 1.36, respectively. This apparent greater oral bioavailability of *l*-MPH in the C57 mouse than in man is in general agreement with plasma results using CD1 mice dosed at 5.0 mg/kg [86] or pregnant rats dosed at 7.0 mg/kg [87] . Further, the extent of accumulation in brain relative to blood will be expected to be less dramatic at 3 h than at earlier time points, especially after oral administration were the decay time course to resemble that of the Sprague-Dawley rat [77].

A primary aim of the present study was to model transdermal MPH – ethanol metabolic interactions. A quarter of the smallest commercially

available MTS patch was used and this delivered a mean dose of approximately 7.5 mg/kg of *dl*-MPH over the 3.25 h wear period based on the difference between drug content before and after application. Though the MTS is not designed to be cut into portions for clinical applications, the *dl*-MPH content in each patch is evenly distributed throughout the patch [16] and required apportioning when using such a small species as the mouse. *dl*-MPH delivery has been reported to occur in a manner directly proportional to the patch surface area in humans [16, 88]. Accordingly, the drug content in the ¼ 12.5cm^2 patches used in the present study was 25% of 27.5 mg, i.e., 6.88 mg. The mean dose of 0.23 mg of *dl*-MPH delivered to the mice (n = 12) over the 3.25 h wear represents 3.3% of the ¼ patch content of *dl*-MPH and ranged from 1.9 - 5.1%. In humans, the uncut 12.5 cm^2 patch size is designed to deliver a mean *dl*-MPH dose of 10 mg over the recommended 9 h wear. This dose represents 36% of the patch *dl*-MPH content, though ranging between subjects from 15-72% [89].

These apparent transdermal *dl*-MPH absorption differences reflect many factors including: (1) the shorter wear time of 3.25 h for the mouse, (2) the faster rate of ester substrate metabolism expected with rodents relative to humans [90], (3) the hair follicle rich shaved skin of the mice opposed to the skin surface of the recommended hip placement in clinical applications, and (4) the potential for a greater *relative* absorption lag time for the 3.25 h wear versus 9 h in humans. In this latter context, the average lag time for

detectable *d*-MPH in plasma after applying MTS to humans is 3.1 h (ranging from 1-6 h) [91]. The above factors notwithstanding, it is recognized that the percutaneous absorption rate for a range of drugs in mice and other rodents has generally been found to be more rapid than in humans or pigs [92].

While the present investigation appears to represent the first MTS study to use mice, previous preclinical studies have shown that shaved mice serve to model transdermal drug delivery [93]. Hairless or nude mice are more typically used for transdermal delivery studies across the range of patch technologies [94], however, the neuropharmacological reference strain status of the C57 mouse provided the justification for its use in investigating *dl*-MPH – ethanol interactions (see Introduction). Maintaining the mice in the metabolic chambers for a total of 3 h allowed for the collection of adequate urine volume for analysis, while still permitting quantification of analytes from blood and brain. In this context, the mean elimination half-life of *dl*-MPH in mice (B6C3F1 strain; 3 mg/kg p.o.) has been reported to be 1.1 h [95], while that of ethanol (2 g/kg i.p.) in C57 mice appears to be approximately 1.3 h [96].

Enantioselective l-EPH transesterification

As with oral dosing in humans [27], coadministration of ethanol and transdermal or oral *dl*-MPH in C57 mice resulted in the enantioselective transesterification of *dl*-MPH, favoring *l*-MPH over *d*-MPH as a substrate. EPH was detectable in the brain, blood and urine of these mice. Selection of

an appropriate species to model esterase-mediated metabolism of *dl*-MPH was an important consideration in our study design. For instance, beagle dogs have been used in pioneering *dl*-MPH metabolism studies [97], and in subsequent toxicokinetic studies [98]. However, esterase-mediated hydrolysis of *dl*-MPH in beagle dogs exhibit the opposite enantioselectivity, preferentially deesterifying *d*-MPH over *l*-MPH [99]. Further, based on both human investigations [27], and the present findings with C57 mice, the enantioselective formation of *l*-EPH with co-administration of *dl*-MPH and ethanol is accompanied by an elevation in *d*-MPH concentrations relative to dosing with *dl*-MPH alone. While *l*-EPH formation was found to be enantioselective, this metabolic pathway was not enantio*specific*, i.e., *l*-EPH concentrations significantly exceeded *d*-EPH values though *d*-EPH was readily detectable and quantifiable in C57 mouse samples following MTS and ethanol, as well as in the urine of animals dosed orally with *dl*-MPH. In humans dosed orally with *dl*-MPH and ethanol, *d*-EPH rarely exceeded 10% of the concentration of *l*-EPH [27].

In potential forensic medicine applications [72], detection of EPH from biological samples could serve as a biomarker to demonstrate combined consumption of *dl*-MPH and ethanol; analogous to the detection of cocaethylene as evidence of cocaine – ethanol coabuse [100].

The high degree of hepatic localization of CES1 compared to its low level of intestinal expression implicates hepatic transesterification as the

primary site of EPH formation after oral dosing of *dl*-MPH [38]. However, when dosing *dl*-MPH by the transdermal route, presystemic esterase metabolism may also occur, as has been reported during percutaneous disposition of ester containing drugs. Transdermal presystemic hydrolysis has been especially associated with the cutaneous fat layer, where methyl ester and ethyl ester containing drugs are reported to be readily deesterified in skin during transdermal transport [92, 101-104]. Some degree of presystemic transesterification of *dl*-MPH to EPH may also occur. In the presence of ethanol, transesterification of methyl esters to ethyl esters has been reported in skin [105]. For instance, the methyl ester methylparaben is rapidly hydrolyzed in skin [102], though in the presence of ethanol hydrolysis of methylparaben is inhibited by competitive esterase-mediated transesterification of methylparaben to ethylparaben in pig [106] or human [107] skin.

As with hepatic esterase substrates, skin esterase activity has also been reported to exhibit enantioselectively, e.g., during prodrug ester activation by hydrolysis [108]. The possibility of cutaneous esterase-mediated biotransformation resulting in transesterification of transdermal *dl*-MPH with ethanol may be favored by the mildly basic cutaneous pH expected at the MTS application site considering the high concentration of *dl*-MPH free base found in MTS [16]. Mild cutaneous basicity has been reported to accelerate the rate of ester xenobiotic hydrolysis. For instance, esterase activity toward

transdermal drug substrates was accelerated at a pH of 8, but was inhibited at the lower pH of 5 [107]. *dl*-MPH is an especially weak organic base even though it contains a secondary aliphatic amine; it exhibits a pKa of 8.4 versus the pKa of 9.6 for the stimulant methamphetamine [109]. This relatively low basicity of *dl*-MPH has been theorized to be a consequence of an intramolecular hydrogen bonding interaction between the amine and the methyl ester carbonyl within the MPH structure [110].

Still considering the potential for some degree of cutaneous EPH formation, in addition to subsequent hepatic metabolism, oral ethanol is rapidly distributed throughout mammalian tissue, and a portion of the non-metabolized dose is excreted cutaneously (sweat), in addition to ethanol excretion by the lungs and kidney [111]. Finally, even oral MPH reaches skin, as demonstrated using commercial sweat patches placed on the back [112].

Significant increases in d-MPH concentrations by ethanol

The concentrations of *d*-MPH in blood, brain and urine were significantly greater in mice dosed with ethanol than those dosed with dH_20. These findings occurred when dosing either transdermally or orally. *d*-MPH elevation following concomitant MPH-ethanol administration was especially pronounced under the conditions used when dosing *dl*-MPH by the transdermal route. However, any direct comparisons between the extent to which ethanol influences either *d*-MPH concentrations or EPH formation as a function of dosing route cannot be reasonably made due to the inherent

disparities of comparing an oral bolus dose of *dl*-MPH with that of the ongoing release of *dl*-MPH from the MTS. It is possible that the elevated *l*-MPH levels associated with transdermal dosing in C57 mice relative to oral dosing could be relevant to the extent to which ethanol elevates *d*-MPH in the course of ethanol interacting with CES1 to form *l*-EPH.

Approximately 50 times more of *l*-MPH reaches the systemic circulation in humans when *dl*-MPH is dosed transdermally than when dosed orally [29], and *l*-MPH is the isomer which enantioselectively serves as a CES1 substrate in the presence of ethanol [27, 38-39, 113-114]. Were ethanol to facilitate *d*-MPH absorption from the MTS through esterase inhibition at the level of the skin and/or liver, the resulting higher drug concentrations, and potentially more rapid rate of absorption of MPH, may influence pleasurable effects [27] of this drug combination, and contribute to additional abuse liability [47-49]. Further, elevated *d*-MPH plasma concentrations pose the potential for adverse or lethal [72] cardiovascular effects [42-43]. In view of the significant influence of ethanol on *d*-MPH concentrations in the C57 mouse model reported here, transdermal *dl*-MPH used to treat adult ADHD may be associated with clinical considerations unique to this route of administration, should drug interaction findings from of this animal model hold for humans.

Figure Legends

Figure 3.1

Enantioselective transesterification of *dl*-MPH to *l*-EPH following concomitant ethanol.

Figure 3.2

Representative GC-MS-SIM chromatogram of *d*-MPH, *l*-MPH and *l*-EPH from a C57 mouse brain extract (upper ion profile). The sample was collected 3.25 h after dosing with ¼ of a 12.5 cm^2 MTS and 3 h after dosing with 3.0 g/kg ethanol by gavage. Enantiospecific analysis used chiral derivatization and a deuterated internal standard (lower ion profile).

Figure 3.3

Calibration plots of spiked mouse urine were used to determine concentrations of MPH and EPH in experimental samples. All $r^2 > 0.99$.

Figure 3.4

Residual MPH from used ¼ 12 cm^2 patches established transdermal dose delivered.

Figure 3.5

Extraction efficiency of unused ¼ 12 cm^2 *dl*-MPH transdermal patches.

Figure 3.6

(A) In mice treated with ¼ of a 12.5 cm^2 MTS for 3.25 h, ethanol (3.0 g/kg, gavaged at 0.25 h) increased total excretion of *d*-MPH in urine and increased *d*-MPH concentrations in blood and brain relative to dH_2O.

(B) In mice gavaged with *dl*-MPH (7.5 mg/kg), concomitant ethanol (3.0 g/kg) increased total 3 h urinary excretion of *d*-MPH, and increased 3 h*d*-MPH concentrations in blood and brain, relative to gavage dosing with *dl*-MPH (7.5 mg/kg) and dH_2O. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3.7

(A) In mice treated with ¼ of a 12.5 cm^2 MTS for 3.25 h, ethanol (3.0 g/kg, gavage at 0.25 h) increased total excretion of *l*-MPH in urine and increased *l*-MPH concentrations in blood and brain relative to dH_2O gavage.

(B) In mice gavaged with *dl*-MPH (7.5 mg/kg), concomitant ethanol (3.0 g/kg) increased total 3 h urinary excretion of *d*-MPH, and increased 3 h *l*-MPH concentrations in blood and brain, relative to gavage dosing with *dl*-MPH (7.5 mg/kg) and dH_2O. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3.8

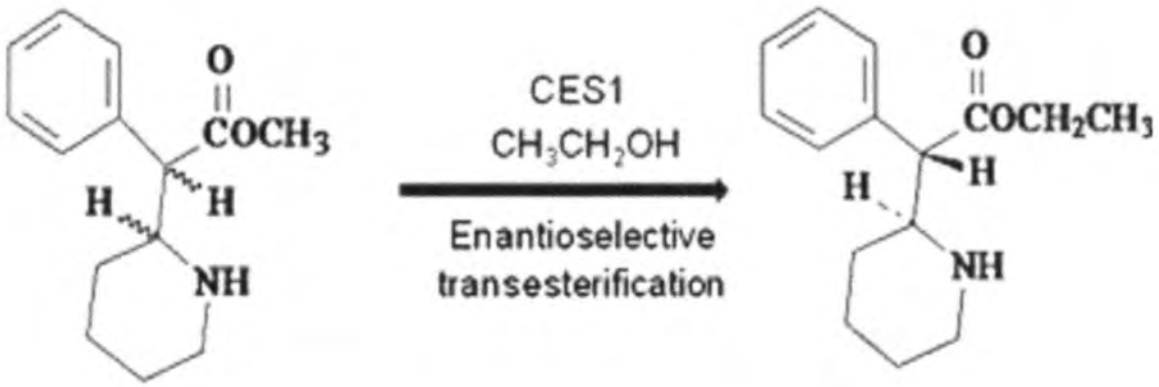
(A) Ethanol (3.0 g/kg, gavage at 0.25 h) and ¼ of a 12.5 cm^2 MTS resulted in enantioselective *l*-EPH formation as quantified in 3.25 h urine, blood and brain.

(B) Concomitant gavage of ethanol (3.0 g/kg) and *dl*-MPH (7.5 mg/kg) resulted in greater 3 h urinary elimination of *l*-EPH than for *d*-EPH. EPH was not detectable (ND) in 3 h blood using dosing regimen. In brain, the mean *l*-EPH concentration was greater, but not significantly (NS) different from that of *d*-EPH. EPH offers the potential of serving as a biomarker for combined *dl*-MPH – ethanol exposure. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

***dl*-MPH and ethanol**	***dl*-MPH and dH_20**
¼ 12.5 cm^2 MTS + 3.0 g/kg ethanol (gavage) n = 8	¼ 12.5 cm^2 MTS + dH_20 (gavage) n = 8
Placebo patch + 7.5 mg/kg *dl*-MPH (gavage) + 3.0 g/kg ethanol (gavage) n = 8	Placebo patch + 7.5 mg/kg *dl*-MPH (gavage) + dH_20 (gavage) n = 8

Table 3.1 Dosing regimens for C57 mice.

Figure 3.1



dl-Methylphenidate *l*-Ethylphenidate

Figure 3.2

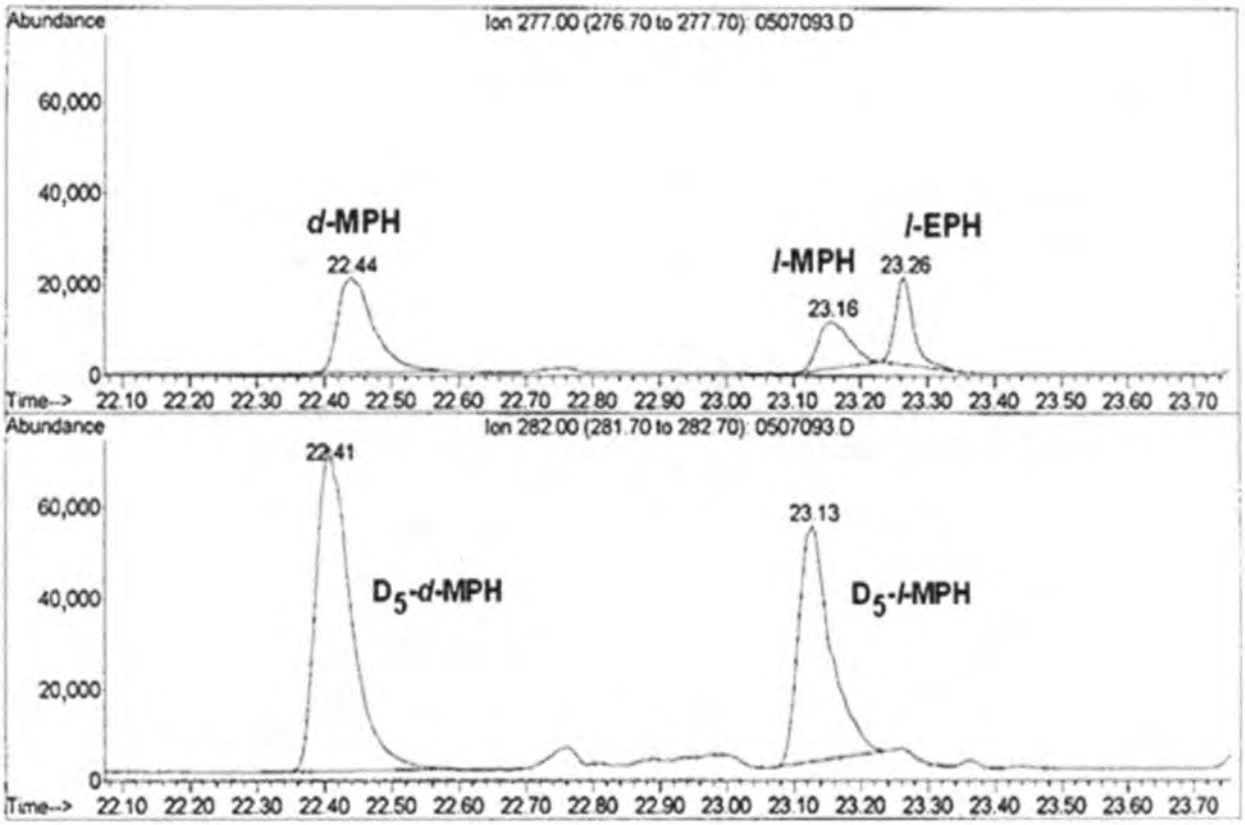

Abundance
Ion 277.00 (276.70 to 277.70): 0507093.D
60,000
40,000
20,000
0
d-MPH
22.44
l-MPH
23.16
l-EPH
23.26
Time-->
22.10 22.20 22.30 22.40 22.50 22.60 22.70 22.80 22.90 23.00 23.10 23.20 23.30 23.40 23.50 23.60 23.70
Abundance
Ion 282.00 (281.70 to 282.70): 0507093.D
22.41
23.13
60,000
40,000
20,000
0
D5-d-MPH
D5-l-MPH
Time-->
22.10 22.20 22.30 22.40 22.50 22.60 22.70 22.80 22.90 23.00 23.10 23.20 23.30 23.40 23.50 23.60 23.70

Figure 3.3

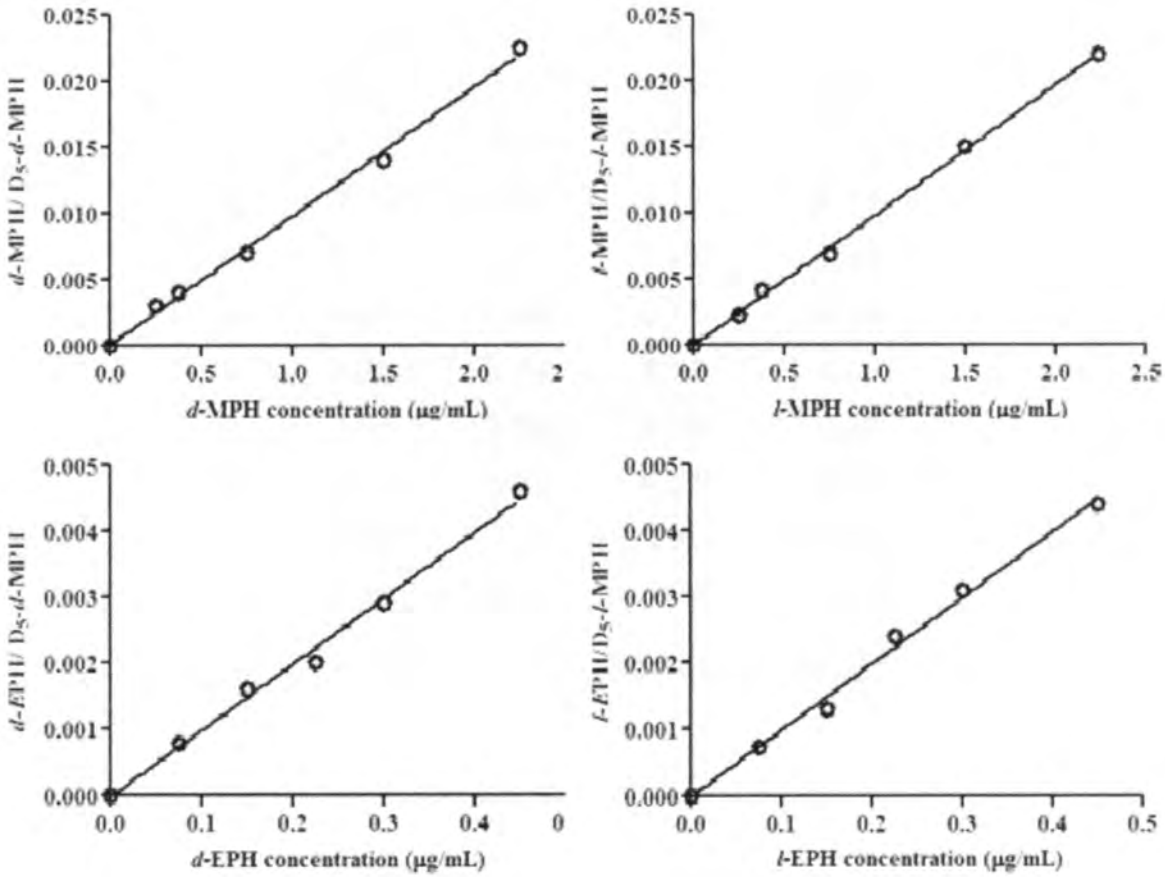

d-MPH/ D5-d-MPH
d-MPH concentration (µg/mL)
l-MPH/D5-l-MPH
l-MPH concentration (µg/mL)
d-EPH/ D5-d-MPH
d-EPH concentration (µg/mL)
l-EPH/D5-l-MPH
l-EPH concentration (µg/mL)

Figure 3.4

ID	d-MPH	l-MPH	total	dose (mg)
1	3.28	3.26	6.54	0.34
2	3.30	3.34	6.64	0.24
3	3.28	3.26	6.54	0.34
4	3.31	3.39	6.70	0.18
5	3.30	3.40	6.70	0.18
6	3.28	3.40	6.68	0.20
7	3.38	3.35	6.73	0.15
8	3.25	3.28	6.53	0.35
9	3.31	3.39	6.70	0.18
10	3.26	3.35	6.61	0.27
11	3.26	3.39	6.65	0.23
12	3.41	3.34	6.75	0.13
			Mean:	**0.23**

Figure 3.5

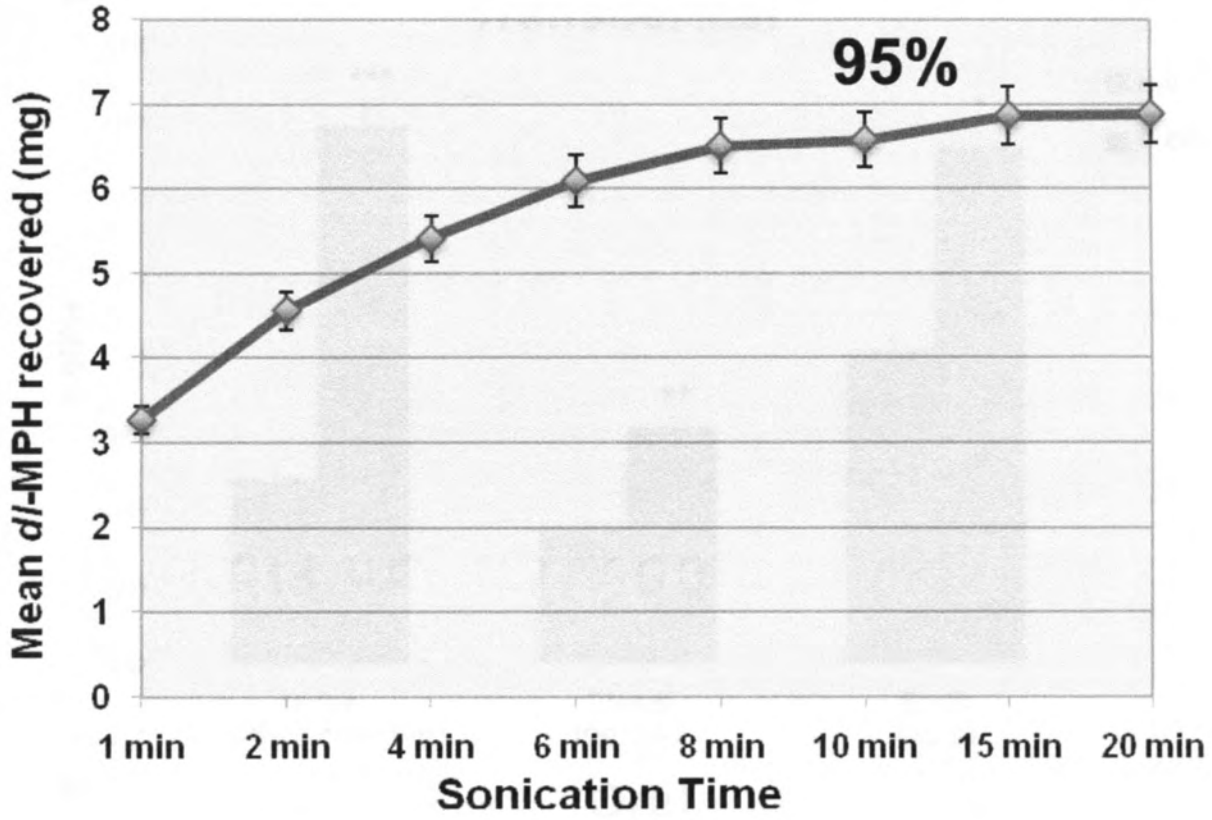

95%
Mean d/-MPH recovered (mg)
0
1
2
3
4
5
6
7
8
1 min
2 min
4 min
6 min
8 min
10 min
15 min
20 min
Sonication Time

Figure 3.6

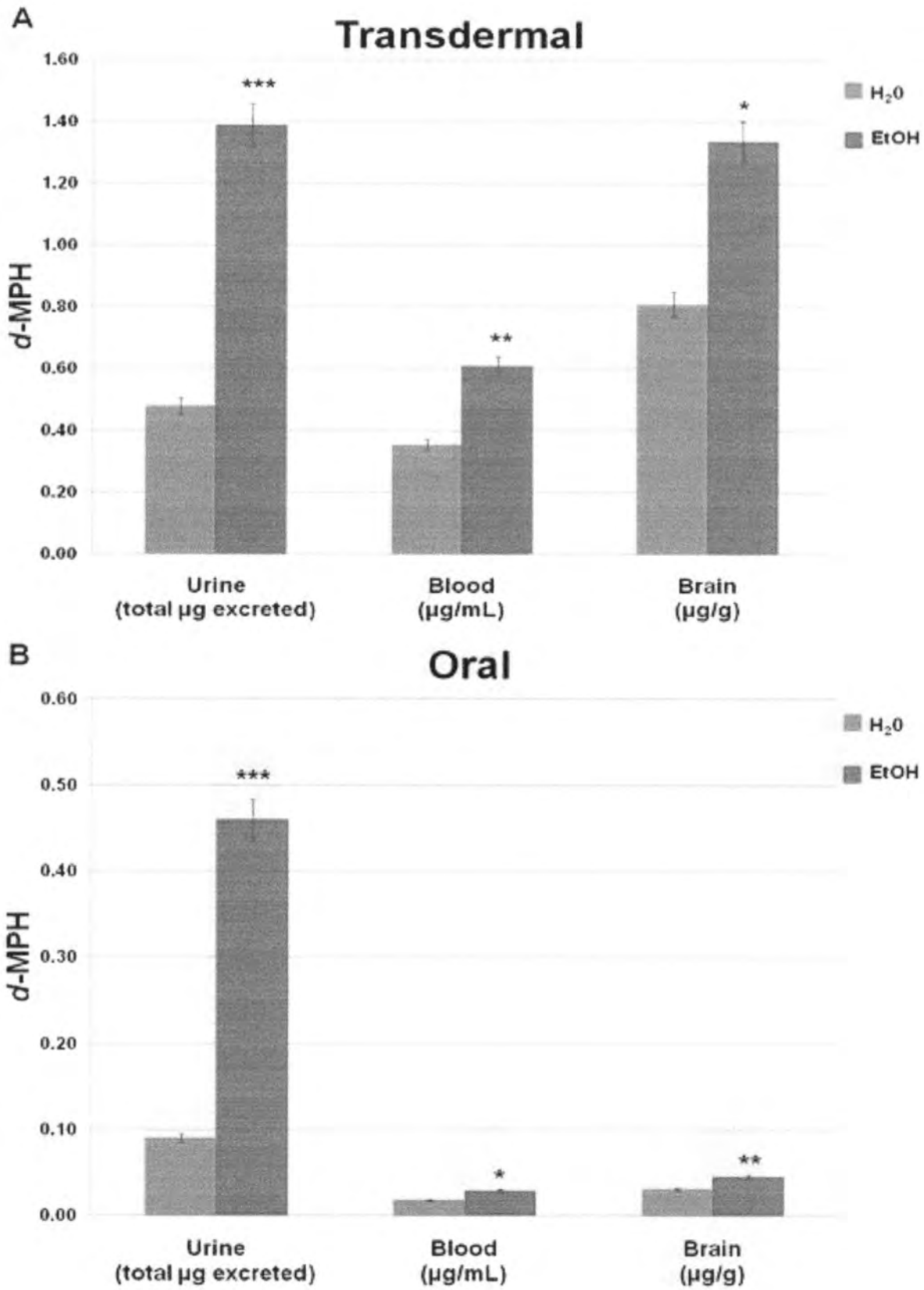

A
Transdermal
d-MPH
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
***
**
*
H20
EtOH
Urine
(total µg excreted)
Blood
(µg/mL)
Brain
(µg/g)
B
Oral
d-MPH
0.60
0.50
0.40
0.30
0.20
0.10
0.00
***
*
**
H20
EtOH
Urine
(total µg excreted)
Blood
(µg/mL)
Brain
(µg/g)

Figure 3.7

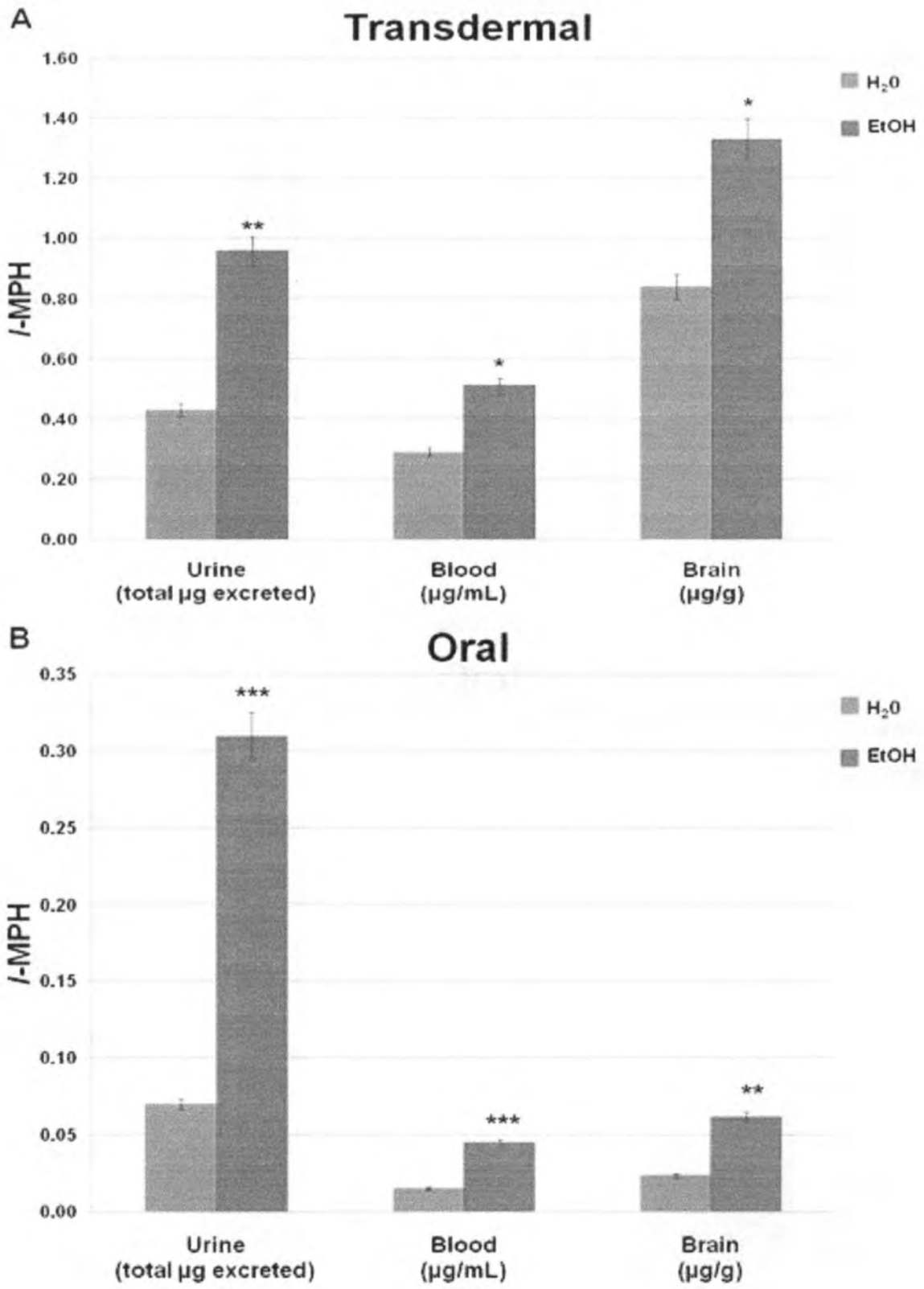
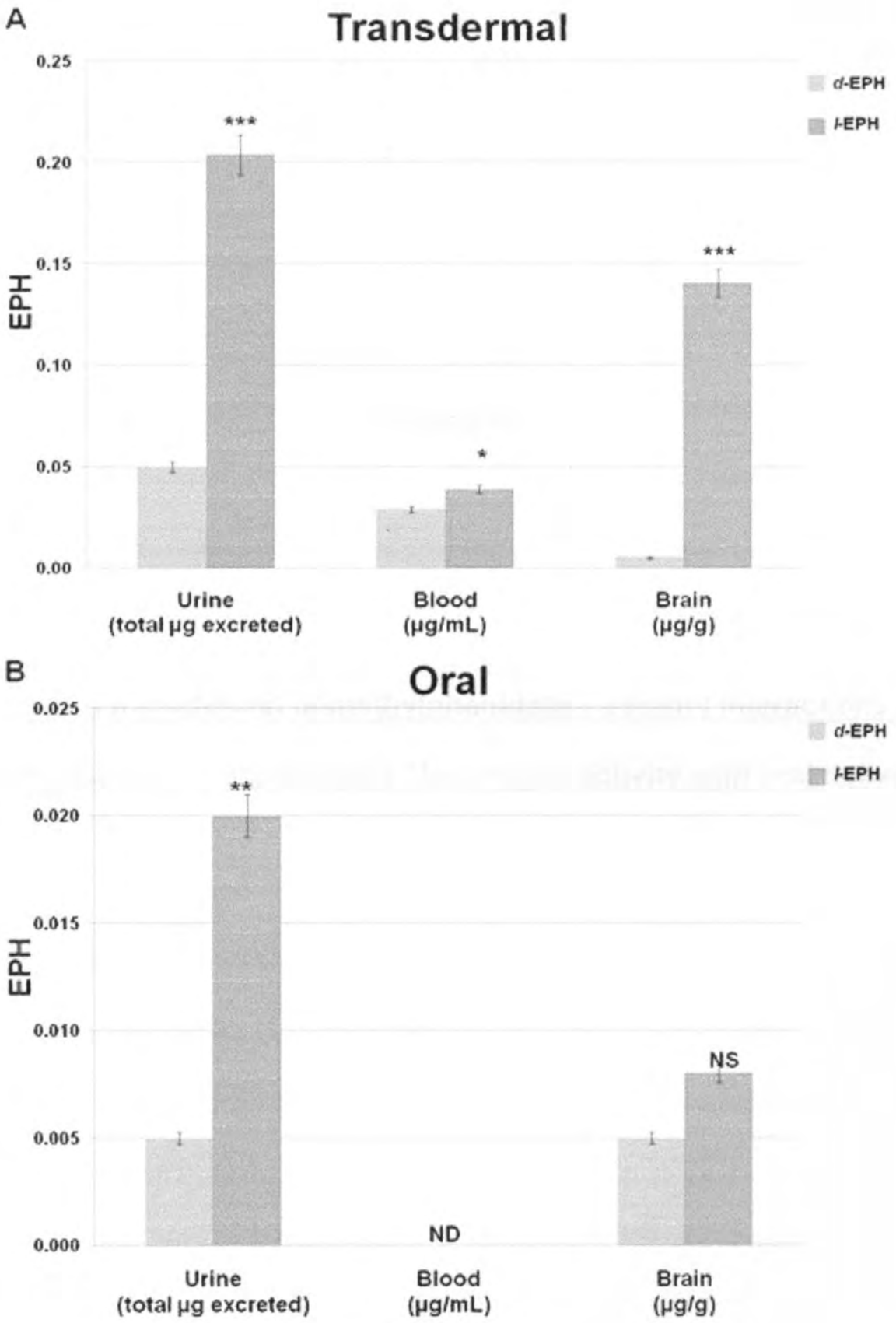

A
Transdermal
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
l-MPH
H2O
EtOH
**
*
*
Urine
(total µg excreted)
Blood
(µg/mL)
Brain
(µg/g)
B
Oral
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
l-MPH
H2O
EtOH
***
***
**
Urine
(total µg excreted)
Blood
(µg/mL)
Brain
(µg/g)

Figure 3.8


A
Transdermal
0.25
0.20
0.15
0.10
0.05
0.00
EPH
d-EPH
l-EPH
***
***
*
Urine
(total µg excreted)
Blood
(µg/mL)
Brain
(µg/g)
B
Oral
0.025
0.020
0.015
0.010
0.005
0.000
EPH
d-EPH
l-EPH
**
NS
ND
Urine
(total µg excreted)
Blood
(µg/mL)
Brain
(µg/g)

Chapter 4

Oral and transdermal *dl*-methylphenidate – ethanol interactions in C57BL/6J mice: Potentiation of locomotor activity with oral delivery

Introduction

The persistent of ADHD into adulthood has been increasingly recognized over the past few decades [4-5]. In a survey, 92% of adult ADHD patients prescribed *dl*-MPH reported concomitant use of ethanol. Further, 100% of individuals who obtained *dl*-MPH through diversion co-abused ethanol [18]. The abuse potential of the *dl*-MPH – ethanol combination is well known in the clinical literature [32-34].

Coadministration of *dl*-MPH and ethanol results in pharmacokinetic and pharmacodynamic drug – drug interactions in humans [27] and in C57 (C57) mice [46, 115]. Ethanol elevates biological concentrations of the pharmacologically active *d*-MPH isomer and yields the metabolic transesterification product EPH [27, 115]. EPH appears to be formed through the actions of carboxylesterase 1 (CES1) [27, 71] which exhibits *l*-MPH substrate enantioselectivity in both the metabolic transesterification and deesterification pathways [37, 39-40] (Figure 4.1). Accordingly, the mean absolute oral bioavailability of *l*-MPH is limited to only 1-3% compared to approximately 30% for *d*-MPH [80]. However, dosing with transdermal *dl*-MPH (Daytrana®) avoids the extensive oral presystemic metabolism and leads to approximately 50 times more *l*-MPH reaching the systemic circulation when compared with oral dosing [16].

The pharmacological significance of dosing route dependent alterations in the relative bioavailability of *d*-MPH versus *l*-MPH was

investigated in the present study using a C57 mouse model in the context of ethanol interactions. The C57 mouse has served as a common reference strain in pre-clinical investigations of psychotropic agents, including the study of *dl*-MPH - ethanol interactions [40, 46, 115], as well as for the behavioral characterization of EPH enantiomers [40-41] . As with humans, C57 mice enantioselectively transesterify *l*-MPH to *l*-EPH [40, 115] (Figure 4.1) as well as exhibit a biphasic excitatory-to-depressant activity profile in response to increasing doses of ethanol [116].

A relatively low intraperitoneal (i.p.) dose of ethanol (1.75 g/kg) has been shown to elevate motor activity for 10-15 min in C57 mice [46]. However, when this dose of ethanol was combined with a sub-stimulatory dose of *dl*-MPH (1.25 mg/kg, i.p.), a potentiation of ethanol induced motor activity occurs. As an extension of this low dose *dl*-MPH - ethanol behavioral study [46], and a C57 mouse dispositional investigation where ethanol was found to elevate blood, brain and urinary *d*-MPH [115] , the following investigation examined the pharmacology of a high, otherwise motor depressive dose of ethanol, combined with a high stimulant dose of oral or transdermal *dl*-MPH. Locomotor activity counts were acquired for 3 h followed by enantiospecific MPH and EPH brain analysis.

The influence of ethanol on the stimulant effects of *dl*-MPH carries special abuse potential and adverse event liability for patients prescribed *dl*-

MPH to treat ADHD, as well as for individuals obtaining *dl*-MPH through diversion.

Materials and Methods

Materials

Ethanol was from AAPER Alcohol and Chemical Co. (Shelbyville, KY; 95%). *dl*-MPH·HCl used for oral animal studies was from Sigma-Aldrich (St. Louis, MO; lot # 118K1052) and 12.5 cm^2 transdermal *dl*-MPH patches (Daytrana®) were from Shire US (Wayne, PA; lot # 2616811; smallest of 4 sizes available). Laboratory tape used to secure transdermal *dl*-MPH or placebo patch (cut Band-Aid® adhesive which closely resembles the texture, adhesion and thickness of the *dl*-MPH patch) was from VWR International (white, 12.7 mm). *dl*-MPH·HCl in methanol (1 mg/mL calculated as free base; Cerilliant, Round Rock, TX) and *dl*-EPH·HCl in ethanol (1 mg/mL calculated as free base, synthesized in-house [41]) were used as the analytical reference standards. Sodium carbonate (Fischer Scientific, Fair Lawn, NJ), n-butyl chloride (Burdick & Jackson, Muskegon, MI), acetonitrile (Mallinckrodt Inc, Paris, KT), (*S*)-N-(trifluoroacetyl)prolyl choride in dichloromethane (1M; Sigma- Aldrich, St Louis, MO), were used for extraction and chiral derivatization. Piperidine deuterated *dl*-MPH·HCl was synthesized in-house [76] and contained approximately 25% of the D_5-isotopolog for SIM monitoring and containing no $D_{0\text{-}1}$-MPH.

Animals

Male C57 mice aged 8-10 weeks (25-35 g) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). They were individually housed in a temperature and humidity controlled colony room on a 12 h light/dark cycle (light: 07.00–19.00 h) with free access to food and water. All experiments were approved by and conducted within the guidelines of the Institutional Animal Care and Use Committee at the Medical University of South Carolina and followed the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80–23, revised 1996).

Locomotor Activity and Analysis

Apparatus

Motor activity was assessed with a Digiscan Animal Activity Monitor system, model RXYZCM(8) TAO with a two-animal option (Omnitech Electronics, Columbus,Ohio, USA). Each activity chamber contained 2 arrays of 16 photo beams spaced 5 cm apart, with eight beams located on the x-axis and eight on the y-axis. One array was located 1.5 cm above floor level to capture horizontal activity and the other was located 6.5 cm above the floor to capture vertical activity of the mice. Stereotypic counts were recorded when the same beam was repeatedly interrupted. Photocells were activated when the photo beams on the wall directly opposite to the cells were interrupted. The Versadat analyzer (Version 2.70-137E) recorded the interruption of each beam and provided the total distance (cm) and vertical activity for each

animal during testing. Each activity chamber was partitioned into 20 x 20 cm quadrants with acrylic dividers to allow simultaneous testing of two mice. Four activity chambers allowed testing of eight mice per session. Each of the activity chambers were enclosed in 90 x 54 x 35 cm sound-attenuated boxes.

Procedures for locomotor activity assessment

On days 1-3, mice were habituated to the motor activity apparatus for 30 min. On day 4 mice were lightly anesthetized with 5% isofluorane for 8-10 min. The hair was clipped with an electric shaver along the abdomen and back, from shoulders to hips. A placebo patch was placed on the lower left hip and secured by laboratory tape over the patch and around the mouse for one full loop to ensure a constant skin interface and to prevent the mice from disturbing the patch. Mice were then gavaged at a volume of 0.02 mL/g body weight with deionized water (dH_20) and placed in the open-field activity chambers for 3 h. On Day 5, mice were randomly placed into 1 of 6 test groups (all with n = 8): placebo patch + dH_20, placebo patch + ethanol, placebo patch + oral *dl*-MPH + dH_20, placebo patch + oral *dl*-MPH + ethanol, transdermal *dl*-MPH + dH_20, or transdermal *dl*-MPH + ethanol. Oral *dl*-MPH was dosed as the HCl salt using 7.5 mg/kg calculated as the free base. This dose was the mean dose absorbed by ¼ patch as established by drug load difference between an unused versus used mouse patch study [115]. Each animal was anesthetized and either a placebo patch or ¼ of 12 cm^2 transdermal patch was placed around the midsection in the same manner as

day 4. They were gavaged at a volume of 0.02 mL/g body weight with either dH_20 or ethanol (3.0 g/kg) and placed in the activity apparatus for 3 h. Following the conclusion of the locomotor activity session, animals were sacrificed and brain samples collected.

The order of treatment groups within each week, and the particular test chamber used to test the different groups was counterbalanced across the entire experiment to eliminate any contribution of possible differences in activity monitors or days of testing to observed effects on motor activity. Total distance and vertical activity were recorded in 5-min bins for the entire 3 h session.

Locomotor Activity Data Analysis

Locomotor activity data in Figures 4.2 and 4.3 was grouped into 20 bins and analyzed using a mixed factor three-way analysis of variance (ANOVA). Oral *dl*-MPH data and transdermal *dl*-MPH data were analyzed separately using a 2(*dl*-MPH dose) X 2(ethanol dose) X 9(TimeBin) design. The between groups factors are *dl*-MPH (dH_20 vs. active dose) and ethanol (dH_20 vs. active dose). The repeated measure is TimeBin. When appropriate, post-hoc comparisons of significant main effects or factor interactions were made using pair-wise comparisons with Bonferroni's correction. Statistical analysis was conducted using PASW Statistics 18 (SPSS Inc., Chicago, Illinois, USA).

Brain MPH and EPH analysis

Enantiospecific analysis of *d*-, *l*-MPH and *d*-, *l*-EPH was conducted as previously described in a recent *dl*-MPH – ethanol disposition report [115] . Briefly, homogenized and alkalinized 1/2 brains were solvent extracted and after chiral derivatization, the samples were injected into a gas chromatograph – mass spectrometer fit with a 5% phenylmethylpolysiloxane column. The trifluoroprolylpiperidyl electron impact fragment ions from analytes and the deuterated *dl*-MPH internal standard were acquired using selected ion monitoring. A range of spiked blank brain calibrators bracketed all concentrations reported as established by linear regression analysis (r^2 > 0.99).

Brain Concentration Data Analysis

A two way analysis of variance (ANOVA) followed by pair wise comparisons using the Student's t-test method was used in the analysis of all data. Samples were analyzed as independent samples and were assumed to have equal variances. Statistical analysis was conducted using SPSS 12.0 (SPSS I.; Chicago, Illinois, USA).

Results

Controls

Mice treated with placebo patches + ethanol (3.0 g/kg) showed significantly less total distance traveled compared to mice treated with

placebo patch + dH_20 over the first 100 min (all *ps<0.05*) and significantly less vertical activity for the entire 3 h (all *ps<0.01*).

Total Distance Traveled

Oral dl-MPH

The total distance traveled data was analyzed by examining changes in horizontal activity across time for the different treatment groups and is summarized in Figure 4.2A. A significant 3 way interaction was found ($F(8,224)= 10.906$, $p < 0.001$). Post-hoc analysis indicated a significant increase in total distance traveled for oral *dl*-MPH + dH_20 compared to the placebo patch + dH_20 for the first 1 h (all *ps<0.05*). Further, total distance traveled for the oral *dl*-MPH + ethanol group was significantly greater than oral *dl*-MPH + dH_20 group over the first 100 min (all *ps<0.5*).

Transdermal dl-MPH

The total distance traveled data was analyzed by examining changes in horizontal activity across time for the different treatment groups and are summarized in Figure 4.3A. The 3 way interaction was not significant for the transdermal *dl*-MPH group. However, the lower level 2 way interactions were significant for TimeBin vs. ethanol ($F(8,224)=5.27$, $p<0.001$) and TimeBin vs. *dl*-MPH ($F(8,224)=28.07$, $p<0.001$). Post hoc analysis indicated a significant increase in total distance traveled for the transdermal *dl*-MPH + dH_20 group compared to the placebo patch + dH_20 group over the 100-180 min time period (all *ps<0.01*).

Vertical Activity

Oral dl-MPH

The vertical activity data was analyzed by examining changes in activity across time for the different treatment groups and is summarized in Figure 4.2B. A significant 3 way interaction was found ($F(8,224)=207.747$, $p<0.001$). Post-hoc analysis indicated a significant increase in vertical activity for oral *dl*-MPH + dH_20 compared to the placebo patch + dH_20 for the first 100 min (all $ps<0.05$). Vertical activity data for mice dosed with oral *dl*-MPH + ethanol and mice dosed with placebo patch + ethanol were significantly decreased compared to the placebo patch + dH_20 and oral *dl*-MPH + dH_20 for the entire 3 h (all $ps<0.001$).

Transdermal dl-MPH

The vertical activity data was analyzed by examining changes in activity across time for the different treatment groups and is summarized in Figure 4.3B. A significant 3 way interaction was found ($F(8,224)=34.935$, $p<0.001$). Post-hoc analysis indicated a significant increase in vertical activity for transdermal *dl*-MPH + dH_20 compared to the placebo patch + dH_20 100-180 min (all $ps<0.01$). Vertical activity data for mice dosed with transdermal *dl*-MPH + ethanol and mice treated with placebo patch + ethanol were significantly decreased compared to placebo the patch + dH_20 and transdermal *dl*-MPH + dH_20 groups for the entire 3 h (all $ps<0.001$).

Brain Drug and Metabolite Concentrations

Oral dl-MPH

The brain concentration of *d*-MPH following oral *dl*-MPH was significantly greater in the animals dosed with ethanol compared to those given dH_20; increasing from 31 ng/g to 51 ng/g (Figure 4.4A; t = 3.92, df = 14, *p<0.001*). Further, in animals dosed with oral *dl*-MPH, concentrations of *l*-MPH were significantly increased from 33 ng/g for animals dosed with dH_20 to 42 ng/g for animals dosed with concomitant ethanol (Figure 4.4A; t = 2.24, df = 14, *p<0.05*). There were no significant differences between the brain concentrations of *d*-MPH and *l*-MPH in animals dosed with dH_20 or in animals dosed with ethanol. Only the *l*-isomer of EPH was detected in animals gavaged with ethanol and was found at a concentration of 10 ng/g (Figure 4.4A).

Transdermal dl-MPH

The brain concentration of *d*-MPH after transdermal dosing was significantly greater in animals dosed with ethanol compared the dH_20 group; increasing from 689 ng/g to 1,294 ng/g (Figure 4.4B; t = 7.38, df =14, *p<0.001*). Further, in animals dosed with transdermal *dl*-MPH, concentrations of *l*-MPH were significantly increased by ethanol, rising from 685 ng/g for animals dosed with dH_20 to 1,210 ng/g for animals dosed with ethanol (Figure 4.4B; t = 7.689, df =14, *p<0.001*). There were no significant differences between the brain concentration of *d*-MPH and *l*-MPH in animals dosed with dH_20, nor in animals dosed with ethanol. Only the *l*-isomer of EPH was

detected in animals gavaged with ethanol and was found at a mean concentration of 130 ng/g (Figure 4.4B).

Discussion

Most oral *dl*-MPH abusers coabuse ethanol [18] and the abuse potential of the *dl*-MPH – ethanol combination is well known in the clinical literature [32-34]. In the present study, C57 mice were used to model pharmacological characteristics of this drug combination to gain insight into the special appeal this drug combination has. The abuse potential of the new transdermal *dl*-MPH formulation has not been investigated in detail at this time. In industry trials, transdermal *dl*-MPH been has reported to produce mild euphoria upon application of 3 or 6 of the 25 cm^2 patches. With the 6-patch application group dysphoria was reported in 42% of the test subjects. It is noted that the FDA requested human testing by the contraindicated application of the patch to buccal mucosa. This tissue surface greatly accelerated *dl*-MPH absorption relative to the normal hip application. Rather than the mean 36% of the patch *dl*-MPH content being absorbed during the recommended 9 h wear, 50% was absorbed in 2 h attached in the mouth (see Patrick et al. 2009).

We have previously reported that a sub-stimulatory i.p. dose of *dl*-MPH in C57 mice potentiates the motor stimulation produced by a low dose of ethanol [46]. The present study used stimulatory oral or transdermal doses of *dl*-MPH (7.5 mg/kg), with or without a depressive dose of ethanol (3 g/kg) to model

dosing route dependent behavioral and dispositional drug interactions as may pertain to both the treatment of adult ADHD patients who use or abuse ethanol and the co-abuse pharmacology of diverted *dl*-MPH and ethanol.

The findings in the present study demonstrate that even a depressive dose of ethanol potentiates a stimulatory dose of oral *dl*-MPH. This potentiation may result from both pharmacokinetic and pharmacodynamic interactions. The 7.5 mg/kg doses of *dl*-MPH used in the present study approaches the highest daily doses found in the medical drug abuse literature, e.g., approximately 10 mg/kg/day intranasally [117] or 29 mg/kg/day intravenously [118] based on 70 kg total body weight (actual weights were not reported). While these doses were reported as total daily doses and the dosing "regimen" undisclosed, the intranasal route and certainly the intravenous routes are expected to result in higher bioavailability than following oral dosing. For instance, only 19% of an oral *dl*-MPH dose reaches the systemic circulation in rats [119] versus approximately 30% in humans [79]. Further, a transdermal dose of *dl*-MPH is absorbed in a prolonged fashion analogous to a multiple dose regimen as the abusers above were likely to have used.

The ethanol dose of 3 g/kg used in this study corresponds to 10 ounces of 80% vodka in a 70 kg human, well within the range of ethanol consumption associated with bingeing. The choice of a 3 g/kg dose allowed comparisons with the urinary metabolites, blood and brain concentrations of *dl*-MPH and *dl*-EPH found in the previous metabolism study [115]. Further, this dose

allowed us to indirectly gauge probable concentrations of ethanol over the course of the present behavioral studies through comparison with literature values in C57 mice. Haseba et al. (2007) found the peak ethanol concentration to be 322 mg% at 0.5 h, declining to approximately 35 mg% 3 h after oral dosing.

Ethanol has been shown to significantly increase the maximum plasma concentration and total exposure to *d*-MPH and *l*-MPH in humans [27], as well as elevate *d*-MPH and *l*-MPH blood, brain and urine concentrations in the C57 mouse [115]. The present brain *d*-MPH, *l*-MPH and *l*-EPH determinations are concordant with our earlier reported brain concentrations [115]. At the 3 h sacrifice time, the mean *d*-MPH brain concentration was 23 times higher in the transdermal group than in the oral dosing group without ethanol. Upon co-administration of ethanol, there was an 88% elevation of *d*-MPH in the transdermal group and 66% elevation following oral *dl*-MPH at the 3 h time point.

At the pharmacodynamic level, the potentiation of *dl*-MPH induced behavioral effects may be based on the mutual influence of these drugs on dopamine, i.e., both *d*-MPH and ethanol have been reported to elevate synaptic dopamine levels. The therapeutic activity of the stimulant *dl*-MPH in the treatment of ADHD prominently involves the reuptake blockade of impulse released dopamine through binding to the dopamine transporter [120] . In our animal model of *dl*-MPH – ethanol co-abuse, the potentiation of stimulatory

effects by concomitant ethanol is consistent with evidence that ethanol releases pre-synaptic dopamine [121-122] as a consequence of upstream GABAergic signaling [123]. Hence ethanol may be increasing extracellular dopamine release, whereby *d*-MPH then blocks a larger dopamine pool from presynaptic reuptake. Further, ethanol-mediated dopamine release significantly increases as the ethanol dose (i.p.) is escalated from 1 g/kg to 2 g/kg to 3 g/kg in C57 mice [122]; the 3 g/kg of ethanol corresponding to the oral dose used in the present study. In this context, we hypothesize that the increased locomotor activity resulting from concomitant oral *dl*-MPH and an otherwise depressive dose of ethanol may reflect a synergistic increase in synaptic dopamine, modeling the accentuation of likeability associated with *dl*-MPH combined with ethanol when compared to *dl*-MPH alone [27].

In humans, the earliest detection of either MPH isomer in plasma after transdermal *dl*-MPH application ranges from 1-6 h [91], unlike oral *dl*-MPH which is readily detectable within 30 min or less [124-125]. The significantly lower total distance traveled of mice dosed with transdermal *dl*-MPH compared to oral *dl*-MPH is likely influenced by a lag phase (latency) in transdermal drug absorption during which time locomotor activity of mice inherently decreases as habituation to the activity chamber occurs. We report here that the lag phase between application of the transdermal *dl*-MPH patch and the onset of pronounced drug-induced motor activity is approximately 100 min in C57 mice. Studies in C57 mice have shown that *d*- and *dl*-MPH

produce dose-related increases in motor activity, while the *l*-isomer produces little or no stimulatory effects [40]. Thus, it is hypothesized that despite significantly higher 3 h brain concentrations of *d*-MPH following transdermal dosing, the total distance traveled of mice did not reach the same levels of early time points following oral dosing due in part to the mice habituating to the chambers and such low activity was not able to rebound to early activity levels found after oral dosing. A further explanation for the attenuated total distance traveled by the transdermal *dl*-MPH – ethanol group could pertain to the induction of stereotypic behaviors associated with such high brain *d*-MPH concentrations, especially in the concomitant ethanol group. This may be supported by the significantly higher vertical activity found at later time points following transdermal dosing. Further, the observation that the stimulant effect of transdermal *dl*-MPH was not potentiated by co-administration of ethanol may relate to the anticipated lag phase in transdermal drug absorption extending well into the elimination phase of ethanol (see [115]. It is noted that the mean elimination half-life of ethanol in C57 mice has been reported to be approximately 1.3 h to 1.5 h [122, 126].

The 13-fold greater *l*-EPH concentration found in the transdermal *dl*-MPH – ethanol group relative to the oral *dl*-MPH – ethanol group is unlikely to directly contribute to the neuropharmacology of this drug combination in view of the inactivity of the *l*-isomer of EPH in vivo or in vitro [40-41]. However, the

l-EPH concentration may indirectly gauge the extent to which *dl*-MPH and ethanol interact with CES1.

The presence of *l*-EPH in the C57 mouse brain samples offers further evidence that this transesterification metabolite can serve as a biomarker for concomitant *dl*-MPH – ethanol exposure [72]. Most importantly, ethanol significantly potentiated oral *dl*-MPH induced stimulant effects and elevated the brain *d*-MPH concentrations in this C57 mouse model. These findings could carry implications for increased abuse liability when ethanol is combined with *dl*-MPH should this model generalize to humans.

Figure Legends

Figure 4.1

Enantioselective de-esterification of *dl*-MPH to ritalinic acid (top right) and transestification to *l*-ethylphenidate.

Figure 4.2

(A) Oral *dl*-MPH + dH_20 significantly increased total distance traveled (*, *p<0.05*) and this effect was potentiated by a depressive dose of ethanol *(+, p<0.05)*.

(B) Oral *dl*-MPH significantly increased vertical activity of mice over the first 100 min (*, *p<0.05*).

Figure 4.3

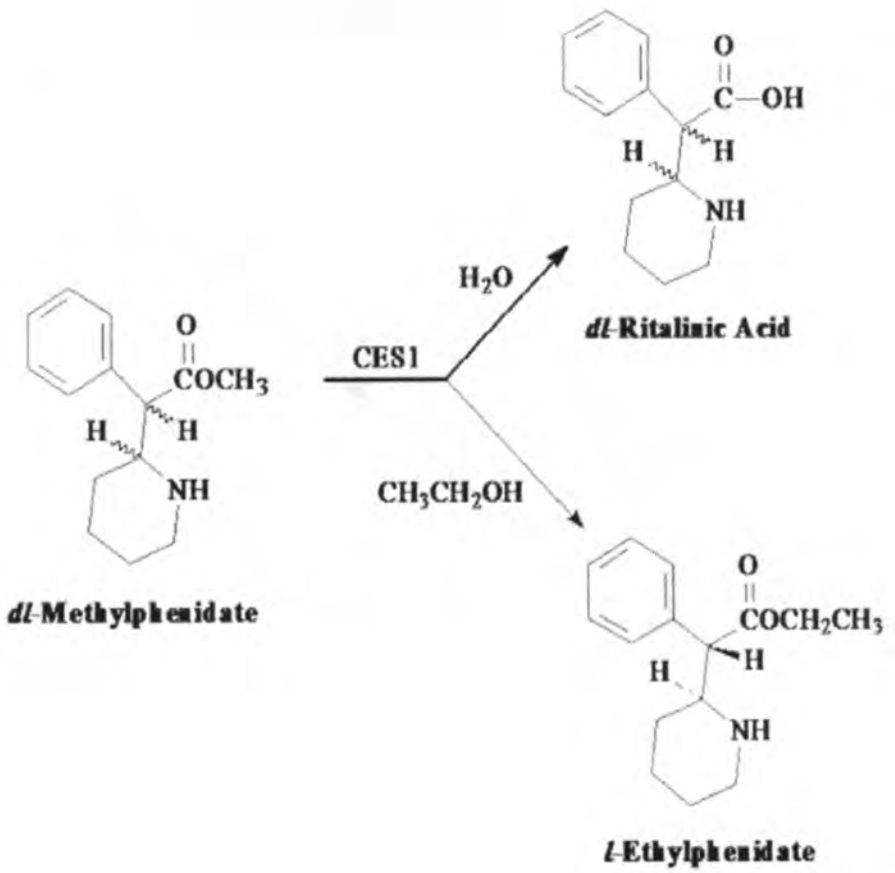
(A) Transdermal *dl*-MPH induced locomotor activity after a lag phase of 100 min (**, *p<0.01*). While this effect was not potentiated by ethanol, transdermal *dl*-MPH + ethanol was significantly greater than placebo patch + dH_20 after a lag phase of 140 min (++, *p<0.01*).

(B) Transdermal *dl*-MPH significantly increased vertical activity of mice after 100 min lag time *(*, p<0.05)*.

Figure 4.4

(A&B) Ethanol significantly increased brain concentrations of *d*-MPH and *l*-MPH relative to dH_20 in mice dosed orally or transdermally (*, *p<0.05;* ***, *p<0.001*).

Figure 4.1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C—OH
H
H
NH
dl-Ritalinic Acid
H2O
CES1
O
COCH3
H
H
NH
dl-Methylphenidate
CH3CH2OH
O
COCH2CH3
H
H
NH
l-Ethylphenidate

Figure 4.2

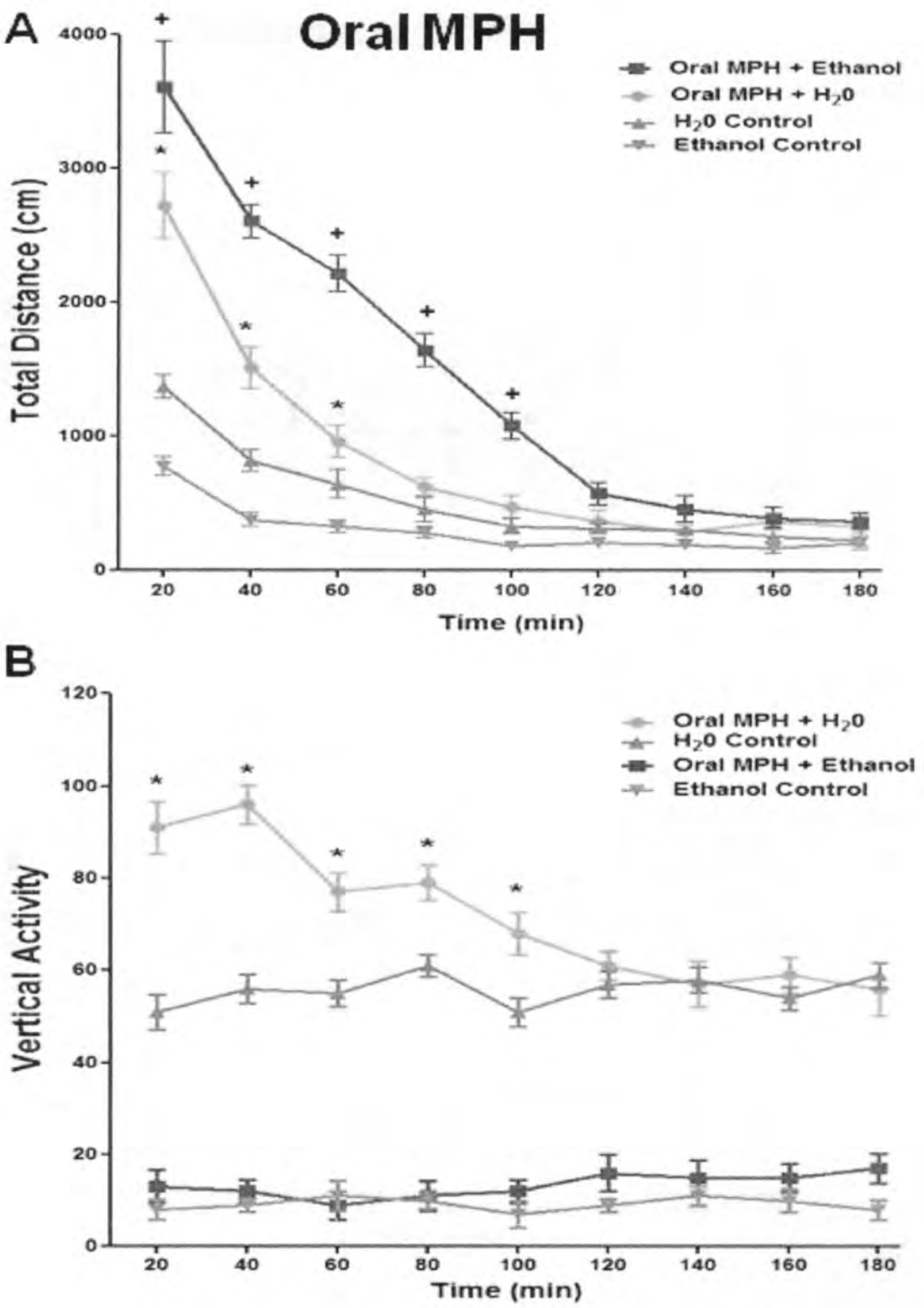

A
Oral MPH
Oral MPH + Ethanol
Oral MPH + H20
H20 Control
Ethanol Control
Total Distance (cm)
4000
3000
2000
1000
0
20
40
60
80
100
120
140
160
180
Time (min)
B
Oral MPH + H20
H20 Control
Oral MPH + Ethanol
Ethanol Control
Vertical Activity
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
Time (min)

Figure 4.3

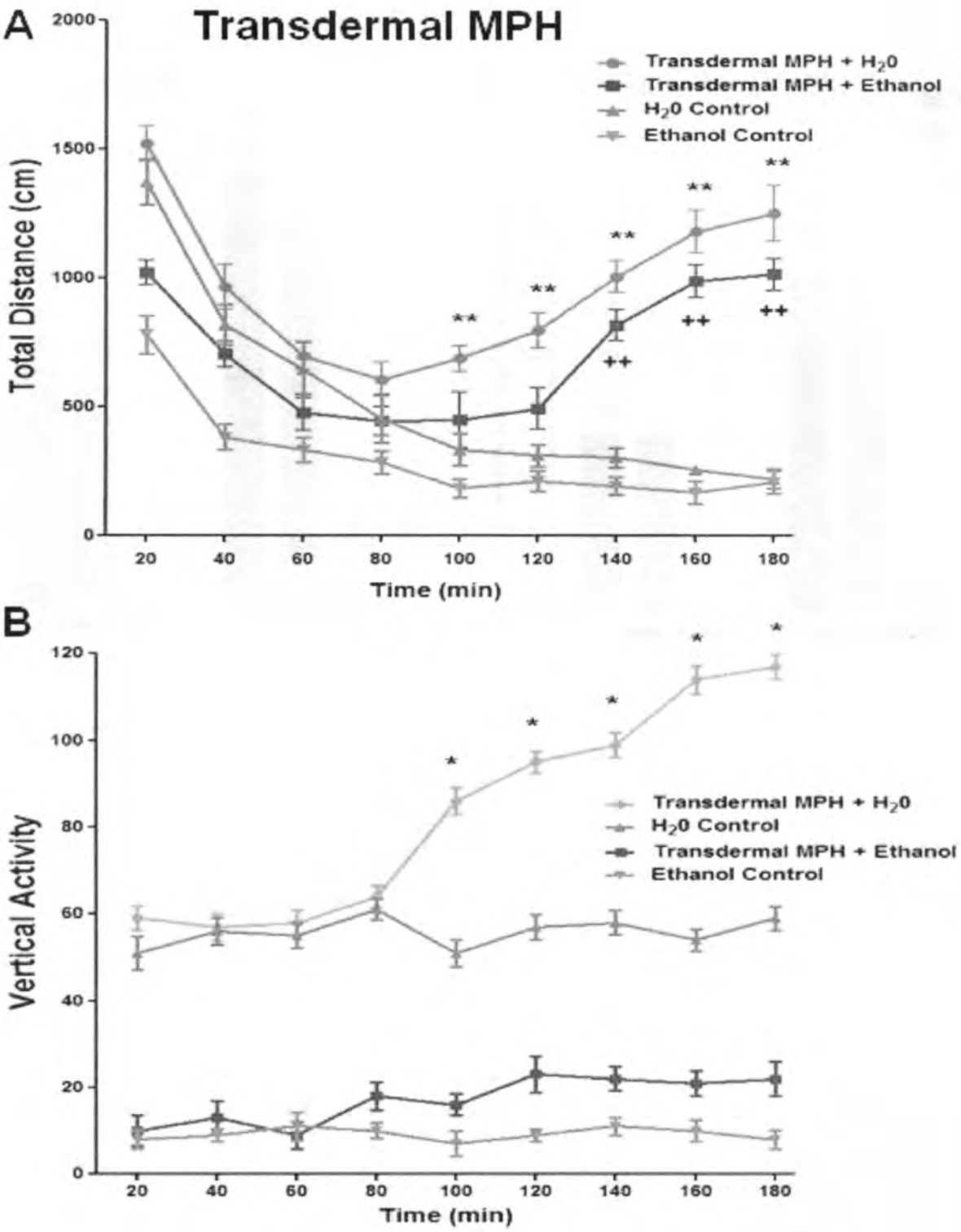
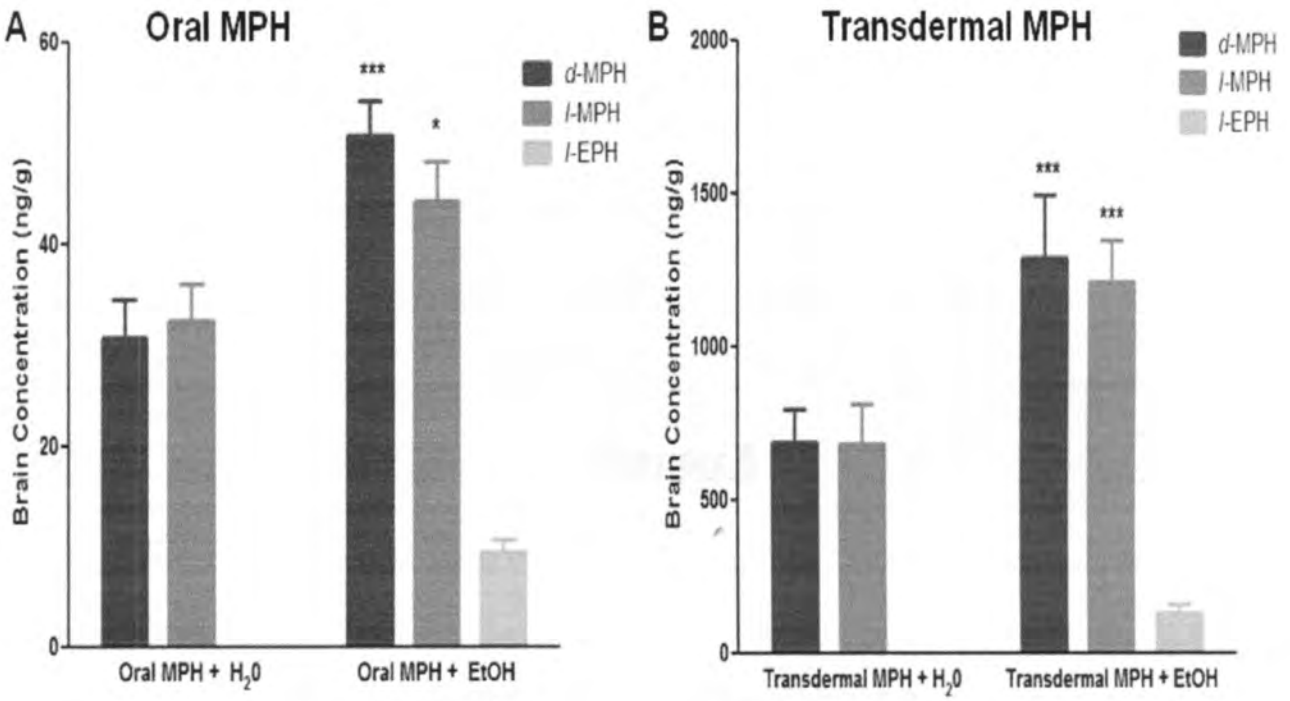

A
Transdermal MPH
Transdermal MPH + H20
Transdermal MPH + Ethanol
H20 Control
Ethanol Control
Total Distance (cm)
2000
1500
1000
500
0
20
40
60
80
100
120
140
160
180
Time (min)
B
Vertical Activity
120
100
80
60
40
20
0
Transdermal MPH + H20
H20 Control
Transdermal MPH + Ethanol
Ethanol Control
Time (min)

Figure 4.4


A
Oral MPH
Brain Concentration (ng/g)
60
40
20
0
***
*
d-MPH
l-MPH
l-EPH
Oral MPH + H2O
Oral MPH + EtOH
B
Transdermal MPH
Brain Concentration (ng/g)
2000
1500
1000
500
0
***
***
d-MPH
l-MPH
l-EPH
Transdermal MPH + H2O
Transdermal MPH + EtOH

Chapter 5

Summary and Conclusions

Summary

The purpose of the experiments in this dissertation was to develop a mouse model to investigate the reward value of *dl*-MPH and the pharmacokinetic and pharmacodynamic interactions with ethanol. Previous studies have used IP injections as a route of administration to investigate *dl*-MPH and ethanol interactions, but the current studies used routes of administration similar to how a normal ADHD patient would consume his or her drugs with a particular focus on the transdermal route of administration for *dl*-MPH and oral administration of ethanol.

In humans, coadministration of *dl*-MPH and ethanol results in a significant elevation of maximum plasma *d*-MPH concentrations (C_{max}) and overall *d*-MPH exposure [27]. Elevated plasma *d*-MPH concentrations increase the potential for adverse cardiovascular events [42-43] due to the fact that the *d*-isomer is responsible for adrenergic pressor effects. In addition to the influence of ethanol on *dl*-MPH pharmacokinetics, the above normal subjects reported an increase in pleasurable effects when combining *dl*-MPH with ethanol [44]. Such positive subjective effects may predispose individuals to greater abuse liability [32-33, 45].

To better understand the pharmacokinetic and pharmacodynamic interactions between *dl*-MPH and ethanol, we first established that C57 mice are a valid model for investigating the reward value of *dl*-MPH. We then used C57 mice to investigate the following research questions: 1) To what degree

does ethanol influence the concentration of enantiomers of MPH and EPH in the brain, blood or urine of C57 mice? 2) What effect does ethanol have on the stimulatory effects of *dl*-MPH in C57 mice? 3) Is there a differential effect of ethanol on C57 mice dosed with oral *dl*-MPH versus transdermal MPH?

Results from Specific Aim 1 confirmed that C57 mice self-administer *dl*-MPH and exhibit robust drug seeking behavior in response to the drug itself and conditioned cues. Previous studies using the Spontaneously Hypertensive Rat as a model for ADHD show that they do exhibit drug seeking behavior [50], however growing evidence suggests they are not an appropriate model for ADHD particularly in their response to first line therapeutics such as *dl*-MPH [51-52]. The C57 mouse is a widely used reference strain for drugs of abuse [40-41, 46, 55] and was chosen as the preferred model for studying *dl*-MPH, particularly with coadministration of ethanol in view of the fact that this strain prefers to consume ethanol [127]. The C57 mouse model has been shown to self-administer cocaine [56] and in the present study has been shown to quickly acquire drug seeking behavior of *dl*-MPH. In this study, self-administration is maintained despite increasingly difficult demands. The reward value of *dl*-MPH is shown through robust drug-seeking behavior despite a two week abstinence period and the lack of drug reinforcement. It is further shown through the maintenance of drug seeking behavior despite removal of condition cues implying that drug seeking behavior in this study was not due to a condition response, but actually due to

the reward value of *dl*-MPH. The results of this study indicate that the C57 mouse is an appropriate model for further studies using *dl*-MPH.

Results from Specific Aim 2 indicated that ethanol significantly altered the pharmacokinetics of dl-MPH in C57 mice, particularly when dosing by the transdermal route. A quarter of the smallest commercially available MTS patch was used and this delivered a mean dose of ~7.5 mg/kg of *dl*-MPH over the 3.25 h wear period based on the difference between drug content before and after application. Though the MTS is not designed to be cut into portions for clinical applications, the *dl*-MPH content in each patch is evenly distributed throughout the patch [16] and required apportioning when using such a small species as the mouse. The mean dose of 0.23 mg of *dl*-MPH delivered to the mice (n = 12) over the 3.25 h wear represents 3.3% of the ¼ patch content of *dl*-MPH and ranged from 1.9 - 5.1%. In humans, the uncut 12.5 cm^2 patch size is designed to deliver a mean *dl*-MPH dose of 10 mg over the recommended 9 h wear. This dose represents 36% of the patch *dl*-MPH content, though ranging between subjects from 15-72% [89]. The apparent transdermal *dl*-MPH absorption differences reflect many factors including: (1) the shorter wear time of 3.25 h for the mouse, (2) the faster rate of ester substrate metabolism expected with rodents relative to humans [90], (3) the hair follicle rich shaved skin of the mice opposed to the skin surface of the recommended hip placement in clinical applications, and (4) the potential for a greater *relative* absorption lag time for the 3.25 h wear versus 9h in humans. In this latter

context, the average lag time for detectable *d*-MPH in plasma after applying MTS to humans is 3.1 h (ranging from 1-6 h)[91]. The above factors notwithstanding, it is recognized that the percutaneous absorption rate for a range of drugs in mice and other rodents has generally been found to be more rapid than in humans or pigs [92].

Our studies with mice dosed with oral *dl*-MPH+d$H_2$0, while being limited to a single 3 h time point for blood and brain sampling, suggest a lower degree of metabolic enantioselectivity relative to humans, whereby the *d*-MPH-to-*l*-MPH ratio for blood and brain were 1.22 and 1.36, respectively. This apparent greater oral bioavailability of *l*-MPH in the C57 mouse than in man is in general agreement with plasma results using CD1 mice dosed at 5.0 mg/kg [86] or pregnant rats dosed at 7.0 mg/kg [87] . Further, the extent of accumulation in brain relative to blood will be expected to be less dramatic at 3 h than at earlier time points, especially after oral dosing were the decay time course to resemble that of the Sprague-Dawley rat [77].

Based on human investigations [27], and the present findings with C57 mice, the enantioselective formation of *l*-EPH following co-administration of *dl*-MPH and ethanol is accompanied by an elevation in *d*-MPH concentrations relative to dosing with *dl*-MPH alone. While *l*-EPH formation was found to be enantioselective, this metabolic pathway was not enantio*specific*, i.e., *l*-EPH concentrations significantly exceeded *d*-EPH values though *d*-EPH was readily detectable and quantifiable in C57 mouse samples following MTS and

ethanol, as well as in the urine of animals dosed orally with *dl*-MPH. In humans dosed orally with *dl*-MPH and ethanol, *d*-EPH rarely exceeded 10% of the concentration of *l*-EPH [27]. In potential forensic medicine applications [72], detection of EPH from biological samples could serve as a biomarker to demonstrate combined consumption of *dl*-MPH and ethanol; analogous to the detection of cocaethylene as evidence of cocaine – ethanol coabuse [100]. In view of the significant influence of ethanol on *d*-MPH concentrations in the C57 mouse model reported here, transdermal *dl*-MPH used to treat adult ADHD may be associated with clinical considerations unique to this route of administration, should drug interaction findings from of this animal model hold for humans.

Results from Specific Aim 3 indicated that that a depressive dose of ethanol potentiates a stimulatory dose of oral *dl*-MPH. This provides additional insight into the reward value associated with *dl*-MPH – ethanol co-abuse (see [18] and [115]). The ethanol -mediated increases in *d*-MPH brain concentrations found following oral dosing, and the potentiated behavioral effects, carry special abuse liability implications for the *dl*-MPH – ethanol combination [18].

The significantly lower total distance traveled of mice dosed with transdermal MPH compared to oral *dl*-MPH is likely due to the lag phase in transdermal drug absorption [16] as well as the fact that locomotor activity of mice decreases over time. It is hypothesized that despite significantly higher

3 h brain concentrations of the *d*-isomer of MPH following transdermal dosing, the total distance traveled of mice (while significant compared to transdermal *dl*-MPH + dH_20) did not reach the same levels of early time points following oral dosing due to the fact that mice had reached such low activity that the rebound effect was not able to reach levels found after oral dosing. A further explanation could be potential stereotypic behaviors associated with such high brain *d*-MPH concentrations and may be reflected in the significantly higher vertical activity found at later time points following MTS dosing. Further, the observation that the stimulant effect of transdermal *dl*-MPH was not potentiated by co-administration of ethanol is interpreted as a lag phase in transdermal *dl*-MPH drug overlapping the elimination phase of the oral ethanol (see [115]).

The presence of *l*-EPH in the C57 mouse brain samples offers further evidence that this transesterification metabolite can serve as a biomarker for concomitant *dl*-MPH – ethanol exposure [72].

The results from these studies in combination with human clinical data indicate that concomitant ethanol significantly alters the pharmacokinetics and pharmacodynamics of *dl*-MPH alone. These interactions have significant implications for abuse liability and toxicity and should be considered before prescribing the stimulant medication *dl*-MPH to an adult population.

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