NEUROPHARMACOLOGICAL SCREENING OF DRUGS OF ABUSE USING FRESHWATER PLANARIANS

By

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ABSTRACT

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Freshwater planarians are invertebrate animals famous for their regenerative ability. They exhibit a body plan similar to vertebrates that include bilateral symmetry with head and tail and most importantly an aggregated of nerve cells in the head containing many neurotransmitters and neurotransmitter receptors commonly found in higher order species. By exploiting these characteristics, this research utilizes the planarian animal model to construct a tool for the pharmacological and toxicological screening of control substances. The ultimate goal of this project is to help forensic science practitioners and lawmakers better understand the pharmacological profile of drugs including the endless stream of novel designer compounds that a major concern to law enforcement and society. Results focus on presenting and discussing both locomotion and behavioral activity exhibited by numerous classes of drugs; explaining expected and deviations from classical behavior, and behavioral descriptions from exposure to new drugs previously not tested in this animal model system.

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CHAPTER 1

Introduction

The Designer Drugs Epidemic

Designer drugs, also known as synthetic drugs or novel psychoactive substances (NPS), are often structural analogs, stereoisomers, or derivatives of a controlled substances chemically produced and specifically designed to mimic the pharmacological effects of drugs of abuse while trying to avoid the law classifiing them as such [1]. Nevertheless, in the United States of America, designer drugs have been gaining traction and becoming widespread in use due to the relative ease at which one can obtain materials for synthesis from both international and domestic sources thus causing difficulty in legislating new compounds once law enforcement encounters them on the street. [1]. In 2016, the National Drug Threat Assessment Summary report by the Drug Enforcement Administration published that the availability of these drugs is increasing in multiple cities and are as common as products sold in gas stations or convenience stores. What is even more alarming is that traditional drug screens do not or cannot test for the vast variety of designer drugs. Even when specific designer drug test are developed new designer drugs are created at a rate that becomes hard to keep up with creating new methodology [2].

In 1986, the Drug Analog Act was passed in conjunction with Controlled Substance Act of 1970 to tackle the designer drug issue, banning substances that exhibit chemical structure similarity to control substances. However, the difficulty in controlling designer compounds was highlighted when JWH-018, a synthetic cannabinoid, hit the street marketed as “herbal spice” [3]. JWH-018 exhibited a dissimilar chemical structure

than the traditional tetrahydrocannabinol yet binds to the cannabinoid receptor as a full agonist, prompting World Health Organization to publish a Critical Review Report on JWH-018 and asking for it to be banned worldwide [4]. Alternatively, the opposite situation can also stand true when drugs with a similar structure can exhibit completely dissimilar effects. One of the prominent examples would be the amphetamines where one of the stereoisomers, dextro-methamphetamine is a controlled substance while levo-methamphetamine is sold over-the-counter as nasal congestion relief [5].

According to National Alliance of Model State Drug Laws, several states have laws providing that:

> "Any [designer] drug that is not currently scheduled under state or federal law but that is similar in structure, or pharmacological effect to a Schedule I or II substance shall be treated as a controlled substance."

Such a statement does not consider what constitutes as pharmacological effect and little is known about synthetic drug toxicology in general. Much less is known about synthetic drug pharmacology and metabolism and sometimes it's impossible to identify what the parent synthetic drug was from detected metabolites especially when mixtures of various designer drugs are administered.

Drug traffickers will work around scheduling actions by modifying chemical formulas to create new, unregulated, and unscheduled drugs [2]. Current legislation is facing the problem that synthetic drugs and human consumption of these drugs create largely unknown pharmacological effects [1]. Lawmakers need to consider such implications on scheduling certain synthetic drugs, whether the substance merits the scheduling based on qualification specified in Controlled Substance Act [1]. Our aim is

to create a screening tool base on the planarian animal model in the hopes of it becoming a low-cost alternative animal model that will allow for pharmacological and toxicological characterization of drugs of abuse, including designer drugs.

Freshwater Planarians

Recently planarians have been identified as a novel animal model to examine the pharmacological and toxicological properties of substances based on the evaluation and measurement of their behavioral responses [6]. Planarians are free-living flatworms of the phylum Platyhelminthes and under the class of Turbellaria. These animals are one of the simplest organisms that exhibit multiple tissue layers, bilateral symmetry and distinctive organs [7]. Planarians are most commonly used in regeneration experiments due to their developmental plasticity. This ability allows planarians to regenerate from damages that are often times fatal to other organisms such as decapitation or dismemberment by the expressing multiple stem cells, known as neoblasts, throughout its body that proliferates lost structures and allows certain species to duplicate asexually [7]. One of the key anatomical features of interest in this study is the organism's possession of a central nervous system (see Figure 1). The planarian nervous system exhibits complex and organized nervous tissues that are generally found in higher organisms; the system consists of bi-lobed cerebral ganglia at the anterior end and two longitudinal nerve cords that underlie the ventral body-wall-musculature. Sensory structures, both photoreceptors and chemoreceptors, are located at the anterior of the animals and when

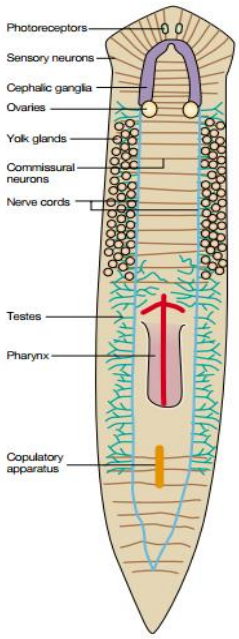

Figure 1 Diagram of the nervous system in freshwater planarians (Newmark and Alvarado 2002)

stimulated, by light or chemicals, will send signals to the cephalic ganglia for process and elicit the appropriate behavioral responses [7]. The invertebrate planarian nervous system plan loosely resembles the vertebrate's in with bi-lobed ganglia resembling a two-lobed brain and two longitudinal nerve cords as two halves of the vertebrate spinal cords. The bilobar brain of the planarians also shows complexity with abundant commissural fibers interconnecting the two halves. Sensory and motor nerves emerge from the cluster location as forms of two solid nerve cords to innervate peripheral structures such as axons, neuropil, and many interneurons to control the muscles and movement [8]. Some suggest that planarians to be the first animal to possess a brain and may be the ancestor of the vertebrate brain. The planarian brain more closely resembles those of vertebrates than those of advanced invertebrates, exhibiting typical vertebrate features of multipolar shape, dendritic spines with synaptic boutons, a single axon, expression of vertebrate-like neural proteins, and relatively slow spontaneously generated electrical activity [8]. Current neurochemical and histochemical studies are also able to provide evidence of the presence of several neurotransmitters and receptors in planarians. Our study hopes to characterize the behavioral response of planarians by exploiting its nervous system to drug exposure under constant light stimulation and create a pharmacological profile based on the chemicals acting on neural transmission. An addition goal of this project was to develop a more automated approach to capturing locomotor activity using accessible software and equipment.

CHAPTER 2

Literature Review

Drugs of Interest

Drugs selected for this study were loosely based on the drugs screened using the Enzyme-Linked Immunosorbent Assay (ELISA) in the Los Angeles Police Department Crime Laboratory. We hope with further research the planarian model could one day become a similar screening tool used in the crime lab. Our hypothesis will build on previous studies which suggested that planarians can be useful as a presumptive diagnostic tool to investigate the effects of drugs and build pharmacological profile base on locomotor activity and behavioral pattern which may help giving insights to explain drugs' reaction on the central nervous system of vertebrates [9].

Opioids

Substances classified as opioids are capable of binding opioid receptors and elicit responses. Opioids are often used in medical treatment to relieve pain and general anesthesia but abused by people due to its psychoactive properties [10]. Opioid abuse, most widely in the forms of morphine or heroin, was criminalized in the United States by the Harrison Narcotics Tax Act of 1914 however, efforts to reduce its abuse has been increased throughout the decade with task forces formed by the Centers for Disease Control and Prevention establishing new prescribing guidelines and recommended dosage [11]. Nevertheless, the recent efforts to combat opioid abuse was curbed due to the rising usage of synthetic opioids according to NDTA Summary. The development of synthetic opioids is not new, for example, heroin is known for its notorious recreational use and controlled by the United States government because of lack of medical use [12]

while some synthetic opioids have medical value such as methadone, developed by scientists in Germany to treat people with opioid dependence as a maintenance therapy [13], or fentanyl, developed by Paul Janssen strictly for use as an anesthetic medicine in surgery [14], but the potential for abuse still exist alongside the beneficiary of medical use. New fentanyl and related compounds beginning to hit the streets are suspected to originate from clandestine labs in China, where the compounds are mass-produced then smuggled into the United States through traditional distribution routes in Mexico. Many new variants of fentanyl are manufactured by Chinese chemists with the purpose of circumventing restrictions that were put on synthetic drugs by the United States [15].

In both the Passarelli et al. (1999) experiment and Buttarelli et al. (2008) review the authors described the presence and response of the κ opioid receptor in planarians. Extensive drug exposure had been done to confirm the receptor presence and eliminate binding of other receptors by using different agonists and antagonists. The increase in dosage also showed opioids influence and behavioral effects are recorded to be able to produce a change in behavioral effects. The careful selection of agonists and antagonists which specifically stimulating different opioid receptor allowed determination of the indirect enhancement of dopamine transmission in which showed similarity in vertebrate animals [9]. We hope to rely on these reports to confirm the binding of designer opioids will have similar effects and through measurable velocity value to create a dosage response. The designer drugs we have selected is Furanyl Fentanyl, known for its intention to be smuggled abroad from China to bypass the laws of the states [15], and also morphine was selected for testing in order to compare and observe the different pharmacological profile at a different level of potency.

Phenethylamine

For the purpose of the experiment, different enantiomers of amphetamines were chosen for testing to observe if the animals are able to differentiate the structural mirror images in this case. Methamphetamine was also tested alongside with a Barbiturate (Secobarbital) to observe and compare the stimulant effect of phenethylamine class. Amphetamines are potent central nervous system stimulants that are often used for the treatment of attention deficit hyperactivity disorder (ADHD), narcolepsy, and obesity. Currently, amphetamine is a control substance Schedule II prescription drug in the United States, due to it has been used as an athletic performance and cognitive enhancer as well as recreationally as an aphrodisiac and euphoriant [16]. Dextroamphetamine (S(+)-amphetamine) and Levoamphetamine (R(-)-amphetamine) are both stimulants however with Dextroamphetamine being the more active stereoisomer out of the two in human [17].

In humans, amphetamines act upon associated receptors to increase monoamine and excitatory neurotransmitter activity in the brain by targeting catecholamine neurotransmitters such as norepinephrine and dopamine [18]. In planarians, it has been shown in reports that amphetamine will induce planarian's locomotor activity, amphetamine acts upon a similar yet more simplified version of endogenous dopaminergic pathways in which will elicit a response in terms of characteristics behaviors dependent on changes in motility to dopamine D1 or D2 receptor [19]. Our study will try to use this behavioral response to comparing the differences between the stereoisomer of amphetamines.

Methamphetamine has been a notorious recreational drug, although it has medical benefits in the United States for the treatment of ADHD and obesity, Food Drug Administration has indicated the limited therapeutic usefulness of the substance and associated it with high potential for misuse [20]. Due to its chemical structure being analogous to amphetamine class, its pharmacological activity is similar yet exhibiting very different effects such as neurotoxicity and shown to damage serotonin receptors in the central nervous system. Methamphetamine has also shown to exhibit a higher potency and a much longer half-life which both contribute to a strong post-acute-withdrawal syndrome that can persist for months [21]. We hope to use methamphetamine as an extreme stimulant to compare its results to a strong depressant such as a barbiturate class drug: Secobarbital.

Benzodiazepines and Nonbenzodiazepine

Benzodiazepines are a class of psychoactive drugs in which the structure showed a fusion of a benzene ring and a diazepine ring. Generally, benzodiazepines enhance the effect of the neurotransmitter gamma-aminobutyric acid at the $GABA_A$ receptor which causes a person to experience mild sedative and sleep-inducing to hypnotic and amnesia effects depending on the dosage [22]. The drug is mainly used to treat anxiety, insomnia agitation, seizures, and muscle spasms due to it also exerts effects such as anxiolytic, anticonvulsant, and muscle relaxant properties [22]. On the other hand, a class of nonbenzodiazepine class drugs also known as “Z-drugs” exerts very similar effects pharmacodynamically to a point almost entirely the same as a benzodiazepine. Z-drugs exhibits the same benefits, side-effects, and risks as benzodiazepines, however, possess a

dissimilar or entirely different chemical structure which is not relatable on a molecular level [23,24].

In planarians, the presence of GABA-like immunoreactivity was present in abundance in the longitudinal nerve cords, lateral nerves, and commissure in the nervous clusters [8]. Recent reports showed an abstinence-induced decrease of planarian locomotor activity after drug exposure and treatment of antagonist, flumazenil, induced withdrawal thus suggesting the possible existence of receptor which can bind agonist or specific site for binding [25].

Although both classes of drug are generally viewed as safe and effective for short-term use, occasional paradoxical effects are not uncommon, aggression or cognitive impairment can occur and shown worsened agitation or cause panic [26]. Long term usage has also shown to have an adverse effect both psychologically and physically as well as showing physical dependence and withdrawal [27]. Z-drugs, in particular, have a history of causing abnormal sleeping behavior that is previously not observed in individuals who had not taken the drug before. We decided that it is of great interest that we conduct the experiment and observe the locomotion effects that the drugs will exert on the planarians for Z-drugs using Zolpidem.

Barbiturates

Barbiturates commonly act as a central nervous system depressant and exhibit a wide spectrum of effects, from sedation to total anesthesia displaying an analgesic effect [17]. Nevertheless, barbiturates do possess abuse potential as one can be physically or psychologically addicted and dependent on. In medical practice, barbiturates have been slowly phased out due to the introduction of benzodiazepines as a lower risk alternative.

Pharmacodynamic of barbiturate shown to act as positive allosteric modulators of $GABA_A$ receptors, and at a higher dose, as an agonist [17,28]. This characteristic allows barbiturates to bind multiple homologous transmembrane packets which are distinctive from GABA or benzodiazepine. This directness can be attributed and reflect a much higher potency than GABA and toxicity [28].

Although barbiturates are still used recreationally, according to National Institute on Drug Abuse, it is slowly declining due to benzodiazepine rising as a cheaper and safer alternative. Nevertheless, barbiturates pharmacological profile still pose a great interest to us as no previous experiment has observed the behavioral or locomotion effect it has on planarians. Secobarbital, one of the more popular barbiturate due to its short acting and intermediate acting, is selected as the main barbiturate for testing.

Cannabinoids

Marijuana is currently the most widely used drug in the United States due to the change in the societal attitude of declining risk of usage as well as several states legalizing its recreational use; marijuana has become a drug of choice for many and has steadily accelerated its appearance in social events [29,30]. Nevertheless, with rising marijuana usage, a new class of designer drugs known as synthetic cannabinoids first went on sale in the early 2000s with some that possess structural similarity and illicit psychoactive properties of cannabis by binding to the same cannabinoid receptors [31].

Two current known cannabinoid receptors, CB_1 and CB_2, are expressed in different areas of the body for human where the CB_1 mainly found in the brain and central nervous system, CB_2 receptors are expressed mainly in the immune system and hematopoietic cells. Delta-9THC, the primary psychoactive ingredient in marijuana

exerts its physiological effects via the central nervous system expressing CB_1 receptor [31]. In planarians, recent literature has shown that exposure to cannabinoid will stimulate the motor activity through an opioid receptor-mediated mechanism despite the simple hierarchy of organization of animal's nervous system, there is evidence of interactions between the distinct neurotransmitter-receptor system in regulating motor behavior when cannabinoid is induced [9,32]. The recent emergence of synthetic cannabinoids that have no structural similarity to delta9-THC has created difficulty when legislating these compounds. We hope to test out MA-CHMINACA a potent CB_1 agonist [33], JWH-133 a potent, selective CB_2 agonist [34] and delta9-THC to see if planarians are able to recognize the compounds base on receptor binding rather than structural similarity.

Hallucinogens and Psychedelics

Another compound of interest is 25I-NBOMe, a psychedelic drug that when used recreationally will alter cognition and perception by acting as an agonist for serotonin receptors [35]. 25I-NBOMe has been reported by users to have very similar effects to Lysergic acid diethylamide (LSD). Recreational use of 25I-NBOMe has been shown to carry a significant risk to both pharmacological and behavioral toxicity, reported by the DEA has led to at least 19 overdose deaths as of August 2015 [36], due to the fact that 25I-NBOMe is still a relatively new substance, and very little is known about its pharmacological risks or its interaction with other substances. With its LD50 value not yet determined and exhibiting the characteristic of a high potency serotonin agonist exerting psychedelic effects [37] has led its current ambiguous legal status, nevertheless many reports it as a designer drug with many using it recreationally starting in 2010

[38,39]. The popularity has prompted some governments to control its possession, production, and sale [36]. Studies have shown that this compound has induced behavioral change in mice, causing it to exhibit head-twitch response [40]. However, no study has been done on planarian's reaction to such novelty drug, and we hope to investigate its pharmacological profile in this experiment further.

Cocaine

Cocaine is currently a widely abused drug as it affects the dopaminergic circuit which is known to involve several types of euphoric behavior in which the users seek [41]. At low dosage cocaine acts as an indirect dopaminergic receptor agonist and inhibits neuronal reuptake of dopamine, however, at higher dosage cocaine also enhances neurotransmitter release [41]. Although mostly used as a recreational drug, topical cocaine can also be used as a local numbing agent to help with the painful procedure in the mouth or nose and has been historically useful as an anesthetic in eye and nasal surgery [42]. However, due to the effect on reward pathway, cocaine is very addictive and remains on control substance schedule for one with higher abusable potential[43].

Recently studies have found that when applying cocaine to invertebrates, specifically planarians, they showed a selective binding of D1 or D2 agonist in which is dependent on the route of administration [41]. Studies have also shown that as the drug concentration increases, the specific behavior is expressed by the planarians from "C-Like Position" to a "Screw Like Hyperkinesia"[41]. These behaviors are especially helpful to quantify and allow us to create a pharmacological profile in combination with locomotive activity. We hope to gain a deeper understanding of such behavior in the planarian system.

CHAPTER 3

Methods and Materials

General Information

Animal preparation

Planarians species used was Dugesia dorotocephala, which were purchased from Carolina Biological Supply (Burlington, NC). Planarians, upon arrival, were transferred to plastic Ziploc® containers with diluted salt water, 0.5 g/L Instant Ocean Salts (Spectrum Brands) with Milli-Q® Ultrapure water (18.2 MΩ) and kept in an environmental chamber at a constant temperature (21° C) in the dark. Animals were allowed 24 to 48 hours to acclimate to the laboratory conditions before any experiment was performed. Experimentation was done typically within one week in the laboratory and animals not being tested or used are fed with fresh commercially available organic beef liver as their water solution changed on a weekly basis. All containers used are washed or soaked with Milli-Q® Ultrapure water to reduce possible plasticizers contamination. When experiments are ready to be performed again, animals are starved for a period of seven days before testing.

Drug preparation

Instant Ocean Salt Distilled Water Solution was first prepared as the source of the solution in which the animals will be placed in for control testing. The solution was first made by mixing 1 liter of Milli-Q® Ultrapure water (18.6 MΩ at 25° C) with 0.500 g of Instant Ocean Salt in a 1-liter plastic bottle container. All bottle containers were regularly washed with Ultrapure water and air dried. Instant Ocean Salt was always stored in a

pressure sealed plastic container to avoid possible moisture contamination, when damped all salts are replaced. Reagents are bought with different companies listed in Table 1.

Table 1 *Source of Reagents*

Reagents	Company	Catalog number
Morphine	Cerilliant©	M-005
Furanyl Fentanyl	Cayman Chemical©	18705
S(+)-amphetamine	Cerilliant©	A-008
R(-)-amphetamine	Cerilliant©	A-049
Methamphetamine	Cerilliant©	M-009
Secobarbital	Cerilliant©	S-002
Delta-9-THC	Cayman Chemical©	12068
MA-CHMINACA	Cayman Chemical©	16421
JWH-133	Cayman Chemical©	10005428
Cocaine	Sigma-Aldrich©	C5776
Zolpidem	Cerilliant©	Z-017
25I-NBOMe	Cerilliant©	C-131

Once planarians, in control conditions (Instant ocean salt solution only), were shown to behave within the range of historical locomotion activity the desired reagent for testing was prepared by first by evaporating the original solvent with nitrogen gas and re-solubilized in 0.5 ml of PEG400 (Polyethylene Glycol). This stock solution was then vortexed and sonicated. The stock solution was then used to create: 2 µg/ml, 5 µg/ml, 15 µg/ml, 30 µg/ml, 60 µg/ml, and 120 µg/ml using diluted salt water solution to create working test solutions. PEG400 had shown to have good biocompatibility and tolerance in comparison to many other solvents tested with the planarians alone in previous experiments to be our choice for the solvent system in these experiments.

Experimental design

The locomotive stimulation revolves around the two light-sensing eye spots connected to the nervous tissue cluster in which under a specific wavelength will stimulate planarian's movement; a recent report has demonstrated that planarians will

change in behavior and experience light phototaxis depending on the wavelength of light utilized [44–46]. Using such characteristic, we can measure the locomotor behavior in response to constant light exposure and constant wavelength thus giving us a controlled behavior to differentiate between locomotor activity under different drug conditions. To be able to simulate such environment we built our instrumentation (see Figure 2 &3) from scratch, drafting it from note papers and finalized our design using SketchUp® for Education computer modeling program. The specifications are on Table 2 as follow:

Table 2 *Specification of Instrumentation*

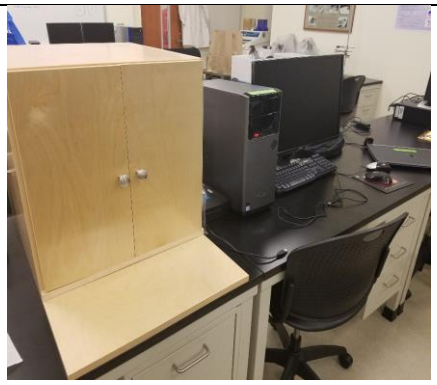

Figure 2. System overview

Figure 3. Light Box interior overview

Computer Hardware
• Operating System Windows 10 64bit • CPU: Intel® Core™ i7-6700 4.0GHz • HD Graphics 530 Intel® H110 • Memory DDR4 2133MHz 8G • Storage 2TB SATA Hard Drive (7200RPM) Hybrid 8GB SSD • Power Supply 300W
Light Box

• Cherry wood exterior
  ○ Pull out top shelf
  ○ Exchangeable bottom plank
• Four HD Cameras
  ○ 30 frames per second
  ○ 15 megapixel
  ○ AdjusTable focus and zoom
• Light Source: LED Panel
  ○ Dimension: 15.63” x 11.81”
  ○ Active area: 13.78” x 9.84”
  ○ Color temperature: 8000K-10000K

Illuminance value is measured with a digital lux meter (Dr. Meter, model: LX1330B) to ensure the correct color temperature is being projected into the Petri dish.

Cameras are also calibrated to ensure distance will reflect pixel conversion accuracy which is done through calculation using NIH ImageJ software (https://imagej.nih.gov/ij/).

To begin testing, animals were initially kept in the dark in the environmental chamber right before use. For each trial, four animals (sized 1.0 – 1.5 cm in length) were randomly selected from their holding container and placed into four different clear plastic petri dishes 5.0 cm in diameter containing 2 ml of the control solution. Locomotor activity was then video recorded for 5 minutes. A control test was first initiated with a minimum of eight animal trials in simple 2 ml Instant Ocean Salt Distilled Water Solution. Animals were used only once and not reused. For drug exposures before subjecting the worms to the drug solutions which had been prepared beforehand the control values were calculated to be within historical velocity normal values. Planarians were exposed to drug concentrations of 2, 5, 15, 30, 60 and 120 µg/ml (see Appendix A #1). Concentration range was chosen because we were primarily interested in determining adverse effect levels for each compound at concentrations relevant for drug dissolution. A minimum of eight animal trials was used for each drug concentration, and animals were used only once and not reused. Following video recording, animals were placed into drug-free distilled salt water and placed back into their dark holding area. Animals were qualitatively monitored immediately following exposure, 1 hour, and 24 hours later for any post-exposure adverse effects. For this monitoring period, we considered adverse effects to include any gross changes in animal morphology including adaptive responses such as excessive mucus production or hyperextension of the pharynx as well as animal mortality. We considered animals to be viable if no obvious adverse effects were present and animals appeared to display typical negative phototaxis to light.

Video Processing and Animal Tracking

The video was acquired using a USB plug and play HD Camera that has 15 megapixels, 1280x960 resolution, and records at 30 frames per second. Resulting video (Figure 4) files were converted into image stacks using virtualdub64 software

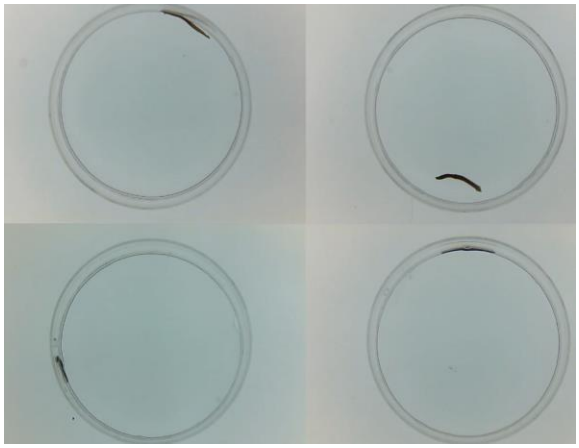

Figure 4 Initial video image produced by the instrumentation in mp4 format

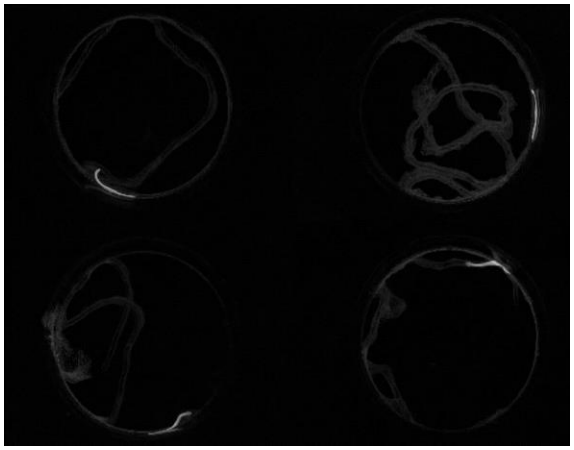

Figure 5 Video image processed by ImageJ to remove background using Image Calculator

(http://virtualdub.org/)with appropriate program plug-ins and further processed using ImageJ software[47]. In ImageJ, file sizes were reduced by changing the file type to 8-bit and resizing the images to 640x480 resolution. The image is then modified with Z-projection function at maximum intensity and using the original image to create stack and difference images by using Image Calculator function (Figure 5). The resulting image stack was then modified to a different threshold using the "maximum entropy" preset, and the MTrack2 plugin (Nico Stuurman) was used to track the worm mass centroid over time. The plugin successfully tracked all animals in the petri dish, and overall frames for greater than 95% of animals tracked. For falsely identified tracks (e.g., solution bubbles) the data was manually analyzed. Only data from the object tracked over the duration of all frames in the image stack and visually identified as the animal were used, or the parameters of the plugin were adjusted only to detect the animal and reprocessed. For each trial the distance traveled in centimeter for 5 minutes under each condition was

calculated using the resulting MTrack2 plugin of x and y coordinates (Figure 6). Macros were created to automate the image processing that both reduced bias in the locomotor

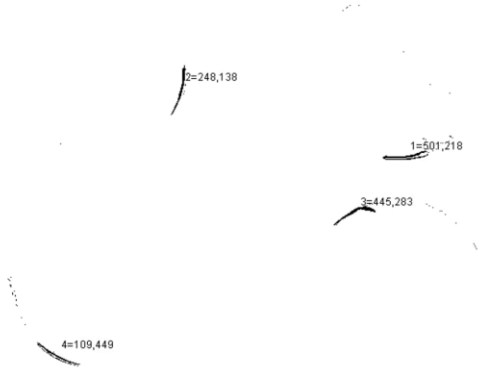

Figure 6 Using ImageJ MTrack2 function to track worms on x & y coordinates

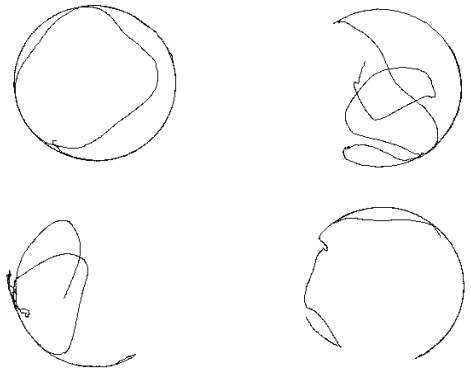

Figure 7 Product of track function showing the total distance traveled in terms of path

activity analysis and overall analysis time. Resulted tracking pixel distance (Figure 7) was first converted to distance in centimeter then divided by time to obtain velocity (see Appendix A #2).

Statistical Analysis

To display the variability of our results, the average velocity (distance traveled in 5 minutes) versus treatment condition was plotted using box and whisker plots where the middle line equals the median, the box extends from the 25^{th} to 75^{th} percentile, and the error bars extend to the lowest and highest values measured. We predicted that the solvents/carriers used in this study would result in an overall decrease in locomotor activity in a concentration-dependent manner. Therefore, comparisons of the average velocity in solvent and drug exposure experiments were evaluated by one-way ANOVA followed by Dunnet's post hoc test (significance criterion, $P < 0.05$). Finally, we constructed X-bar, and R control charts using the mean and standard deviation of the distance traveled of distilled salt solution only exposed replicates from each consecutive day of analysis. Upper and lower control limits, (UCL and LCL) were calculated using

factors associated with n=5 replicates and plotted. The range and sample mean was evaluated to determine the overall reproducibility of the locomotor assay.

Behavioral Analysis

Observation will first be made on the control group to determine "Normal Behavior" which should be consistent and reproducible in diluted salt water solution and under the same environmental conditions. Once the normal behavior is determined is it then compared and contrasted with planarians that are under drug exposure. Behavioral changes that deviate from the normal behavior will be labeled as "Abnormal Behavior" these behaviors should show consistency within the same concentration. If a different abnormal behavior arises in a new concentration, it should also be noted as a secondary abnormal behavior and further tabulated together. All behaviors observed are manually counted by watching the video recording and data will be compiled in a graph form for better visualization to quantify.

CHAPTER 4

Results

Reproducibility

In a forensic toxicology lab setting, control charts are often used to monitor the reproducibility of a method. Our locomotor assay procedure was considered reproducible if the variation between control experiments with diluted salt water solution only replicates within calculated lower and upper control limits. For the R-chart (Figure 8) the Upper Control limit (UCL) = 45.23 cm and R-bar = 21.39 cm. For the X-bar chart (Figure 9): Lower Control Limit (LCL) = 30.78 cm, Upper Control Limit (UCL) = 55.46 cm and our X-bar = 43.12 cm. As demonstrated in the Figures below, consecutive control runs that spanned over a period of two years fell within these limits with our grand mean of 43.12 cm. Both charts showed that the method is a precise test procedure since the results showed similar locomotor activity between tests performed on different dates with different operators under the same condition. The behavioral pattern that arises from control testing showed that light-stimulated-planarians generally move in a circular fashion and follow around the circumference of the petri dish with a constant velocity and occasional direction change.

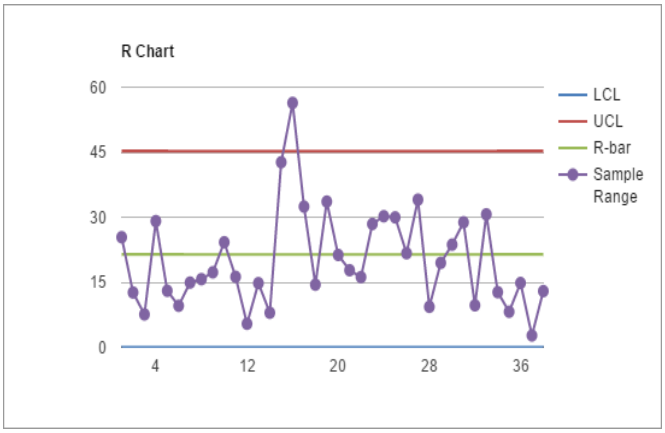

Figure 8 R-Chart with Sample data plotted

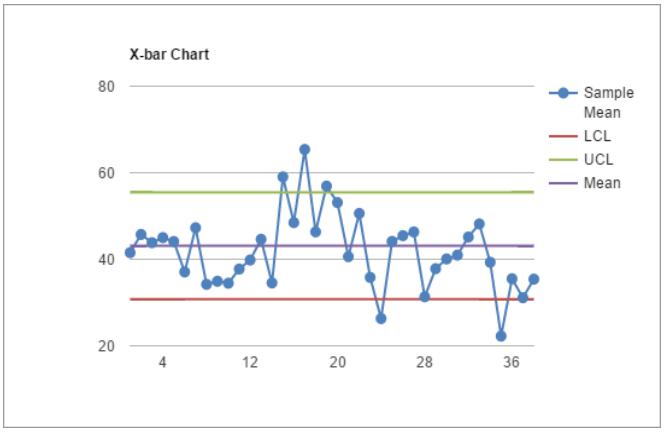

Figure 9 X-bar Chart with Sample data plotted

Statistical analysis results are obtained through Graph Pad Prism 4.02 computing both one-way ANOVA and Dunnett's test (see Appendix B). A significant difference between the control and concentration is indicated by the number of asterisks with one asterisk showing P value less than 0.05 and two indicating P value less than 0.01.

Opioids

Morphine and Furanyl Fentanyl were solvated in pure PEG400 to create dilutions for testing. Dissolution was assisted by sonication and gentle heating.

Data in Tables are plotted in Figure 10 and Figure 11

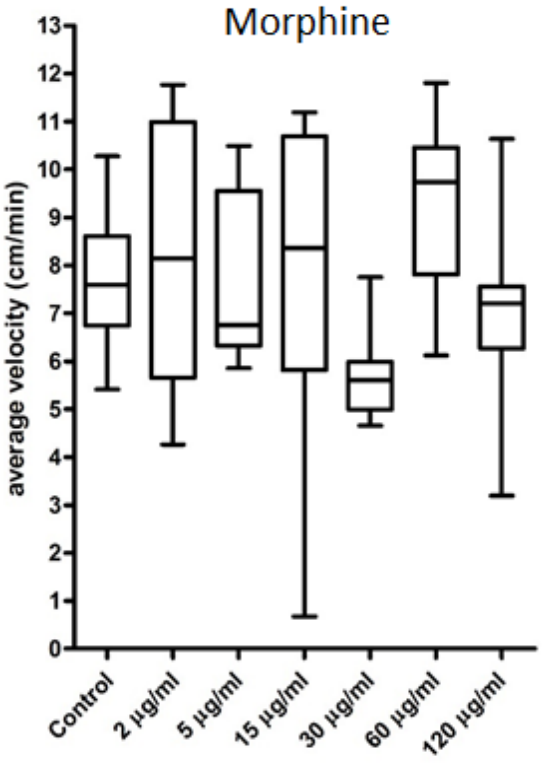

Figure 10 GraphPad Prism Result for Morphine

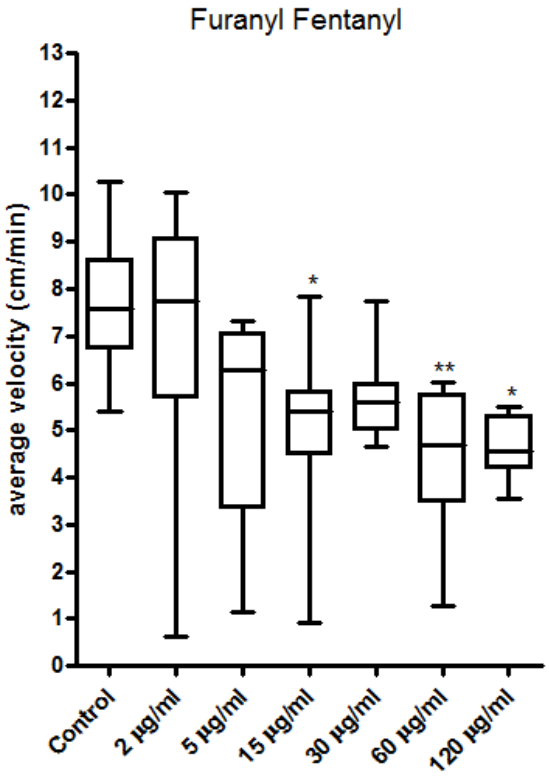

Figure 11 GraphPad Prism Result for Furanyl Fentanyl

Behavior analysis was performed according to literature and counted manually by observing the recorded videos and plotted as tabulated with the prominent abnormal behaviors which include: Snake-Like Motion (SLM) where they form an S-shape in place

or snaking out of the dish. Screw-Like Hyperkinesia (SLH) showing extreme twisting on multiple occasions, and C-Like Position (CLP) that also exhibit curling up. Post-24-hour observation had shown that most of the planarians survived the exposure to morphine across all concentration and Furanyl Fentanyl at low to medium concentrations. Nevertheless, planarians exposed to morphine and Furanyl Fentanyl at higher concentration, 60 µg/ml and 120 µg/ml respectively, lysed and died from the exposure.

Table 3. *Behavioral count for Morphine (M) and Furanyl Fentanyl (F)*

Conc. / Behavior	2 µg/ml		5 µg/ml		15 µg/ml		30 µg/ml		60 µg/ml		120 µg/ml	
	M	F	M	F	M	F	M	F	M	F	M	F
SLM	3	6	4	7	5	7	8	8	8	7	8	8
SLH	0	2	1	6	2	7	5	8	7	7	8	8
CLP	0	0	0	1	0	3	0	5	0	8	2	8

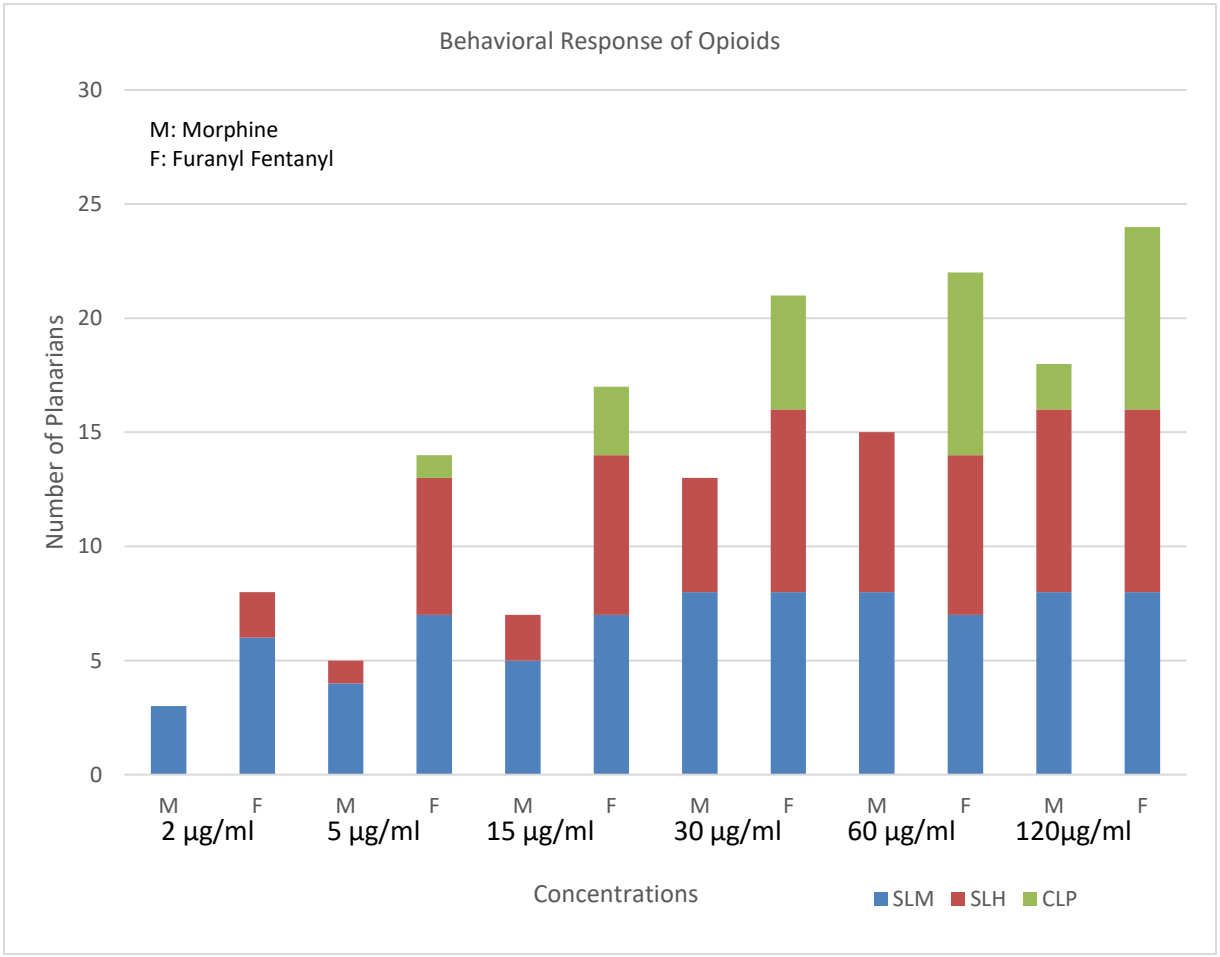

Figure 12 Plotted result of opioid behavior analysis

Amphetamines

S(+)-amphetamine in 5 ml methanol and R(-)-amphetamine in 5 ml methanol were successfully nitrogen evaporated and reconstituted by solvating in pure PEG400 to create dilutions for testing. Dissolution was assisted by sonication and gentle heating. Due to the limited amount of animals available at the time R(-)-amphetamine (Figure 13) was only tested for a total of five different concentrations instead of six. While S(+)-amphetamine was tested with the normal amount of planarians (Figure 14).

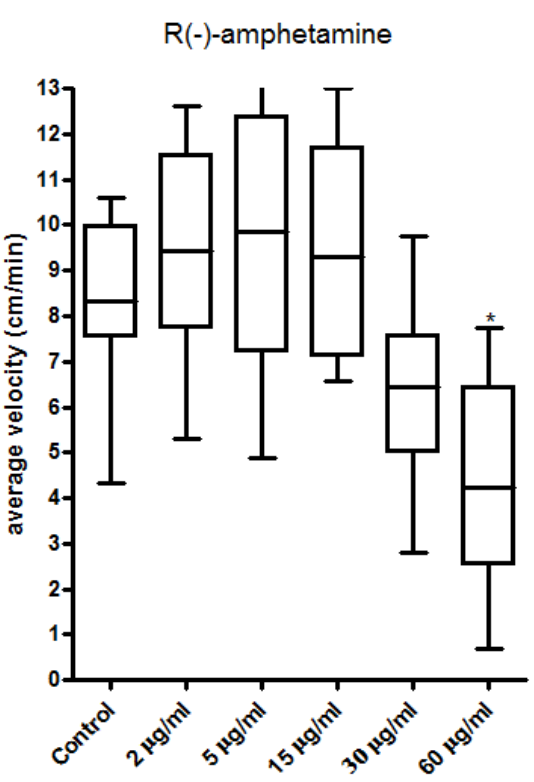

Figure 13 GraphPad Prism Result for R(-)-amphetamine

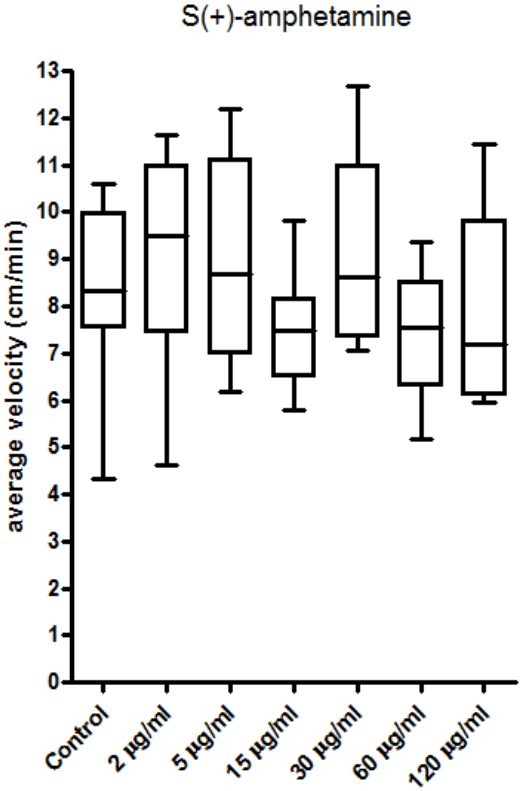

Figure 14 GraphPad Prism Result for S(+)-amphetamine

Behavior analysis was performed by observing the recorded videos. R(-)-amphetamine had shown the following behaviors: Exaggerated Head-swings (EHS) where sudden turn and change in direction, C-shaped Twisting (CST) in which the planarian was circling in place while twisting or curling, and Head-twitch movement

(HTM) where the anterior portion of the planarians will twitch significantly. At higher concentrations planarians began to curl, contract, and some completely stop moving in which we will describe it as Curling Contraction (CC). S(+)-amphetamine had shown similar characteristics, however, varies with different concentration. Many of the planarians seem to crawl out of the petri dish when exposed to the drugs in which is observable in a high concentration of R(-)-amphetamine and low concentration of S(+)-amphetamine. Post-24-hour observation has shown no significant change in planarian activity or mortality. All described behaviors are counted manually, plotted, and tabulated as below in Table 4 and Figure 15:

Table 4. *Behavioral count for R(-)-amphetamine (R) and S(+)-amphetamine (S)*

Conc. / Behavior	2 µg/ml		5 µg/ml		15 µg/ml		30 µg/ml		60 µg/ml		120 µg/ml
	R	S	R	S	R	S	R	S	R	S	S
EHS	6	8	8	8	8	8	1	7	4	8	8
CST	6	2	8	2	8	5	1	6	4	8	8
HTM	6	8	8	8	8	8	2	7	4	8	8
CC	0	0	0	0	0	0	3	0	5	0	0
Crawled Out	0	4	0	2	0	1	2	1	2	2	1

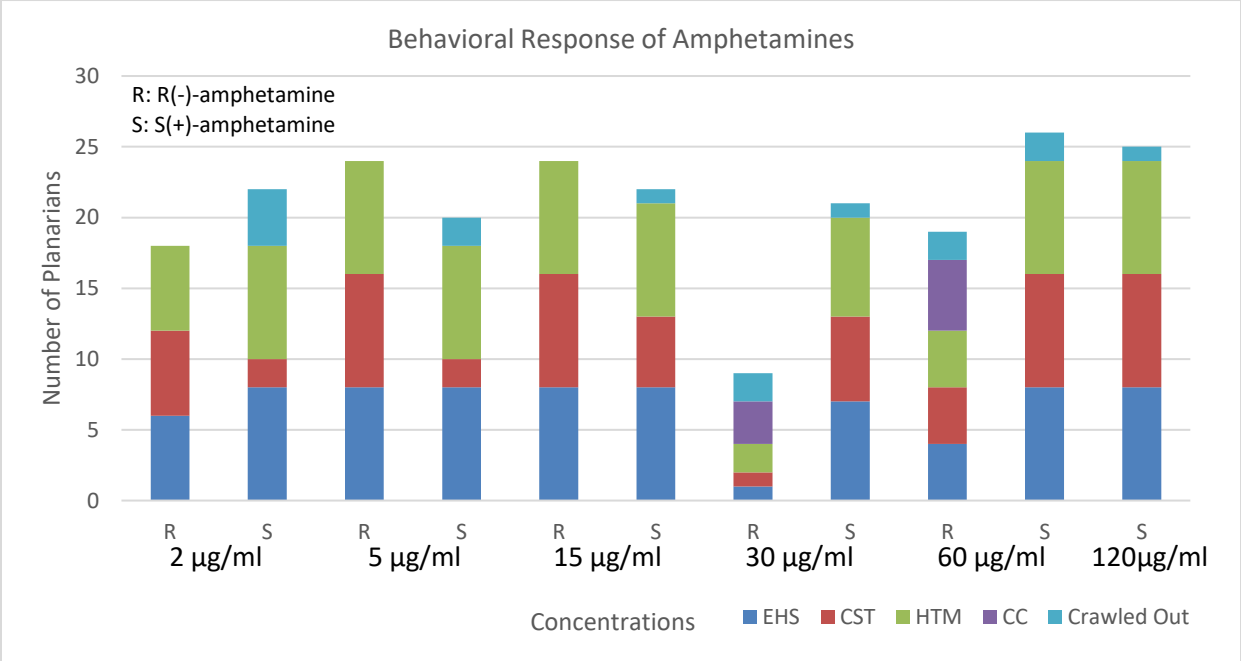

Figure 15 Plotted result of Amphetamines behavior

Benzodiazepine-like: Nonbenzodiazepine

Zolpidem in 5 ml methanol was successfully nitrogen evaporated and reconstituted by solvating in pure PEG400 to create dilutions for testing. Dissolution was assisted by sonication and gentle heating. Locomotor activity is shown in Figure 16.

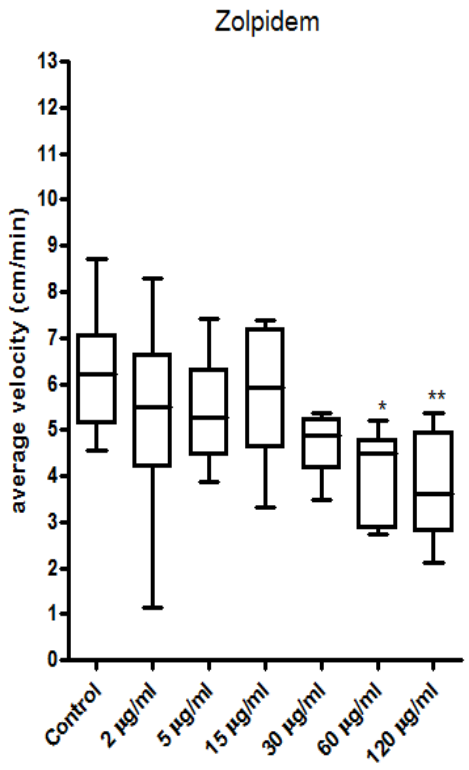

Figure 16 GraphPad Prism Result for Zolpidem

Behavior analysis was performed by observing the recorded videos. Lower concentration Zolpidem had shown a slight twitching anterior movement of the planarian but did not display the behavior consistently through the rest of the concentration. It is worth mentioning that a couple of the planarians also showed contracted body and remained still for the duration of the recording showing a characteristic of hyperkinesia in early concentration. However, the amount was not significant enough to be included in the tabulated results. Most common behaviors observed during the Zolpidem drug exposure showed planarians would start their movement with CLP and progressed into a Twitching and Curling in place behavior (TAC) in which the planarians would display exaggerated twitching and curling movement but remain in the same location while doing so. Similarly, at higher concentrations, behavior: Extending and Contracting in place (EAC) also shown planarians to be remaining in place while displaying an extension of its body dorsally and

contracting right after the extension. Post-24-hour observation revealed no significant change in planarian activity or mortality. Observations were formulated in Table 5 and Figure 17 below:

Table 5. *Behavioral count for Zolpidem*

Conc. / Behavior	2 µg/ml	5 µg/ml	15 µg/ml	30 µg/ml	60 µg/ml	120 µg/ml
CLP	4	6	5	8	8	8
TAC	0	1	3	7	8	8
EAC	0	0	0	3	5	8

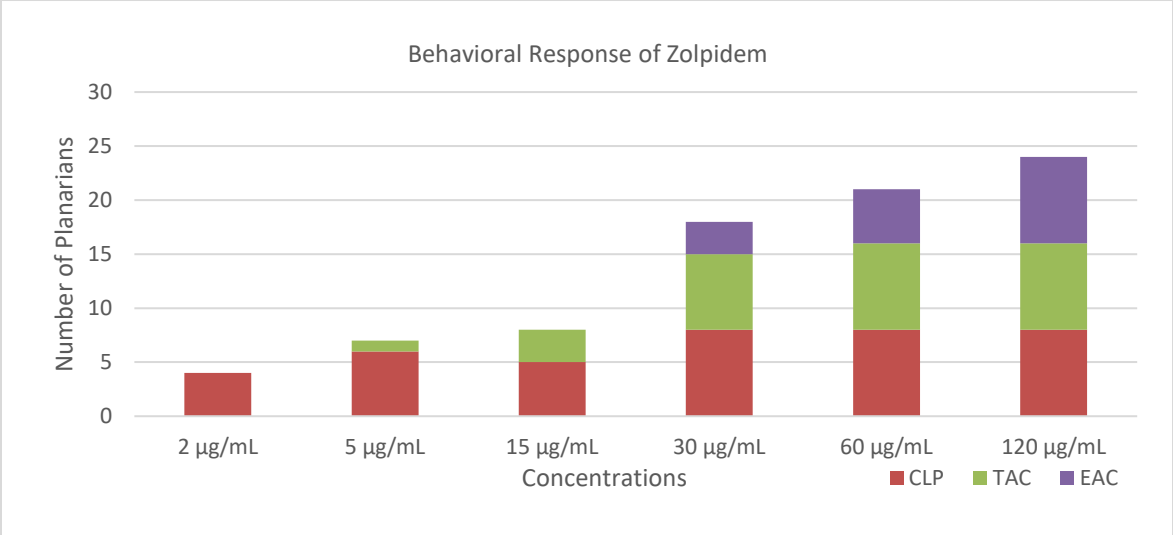

Figure 17 Plotted result of Zolpidem behavior

Barbiturate vs. Methamphetamine

Secobarbital in 5 ml methanol and Methamphetamine in 5 ml methanol were successfully nitrogen evaporated and reconstituted by solvating in pure PEG400 to create dilutions for testing. Dissolution was assisted by sonication and gentle heating.

Behavioral analysis was performed by observing the recorded videos. Methamphetamine displayed very similar behavioral profile to amphetamines showing EHS, CST, HTM at early concentrations and hyperkinesia-like C-shape curl (HLC) in higher concentrations. Secobarbital, on the other hand, has shown to have similar EHS and HTM but with a much more exaggerated hyperkinesia-like C-shape curl throughout

concentrations. Post-24-hour observation had shown mortality in higher concentration of both methamphetamine (at 60 µg/ ml and 120 µg/ml) and Secobarbital (at 30 µg/ ml, 60 µg/ ml, and 120 µg/ml). Some of the behavioral worth mentioning, but not common enough to skew the data. The purpose of the testing was to distinguish the effects of a stimulant (Figure 18) and a depressant (Figure 19). Therefore, we devised only to count and tabulate data involving the hyperkinesia profile (Table 6 and Figure 20) for both

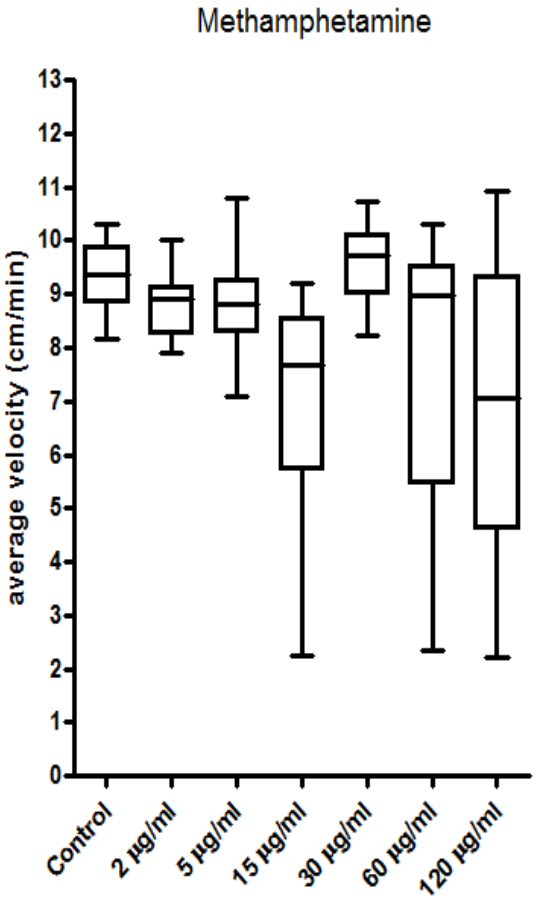

Figure 18 GraphPad Prism Result for Methamphetamine

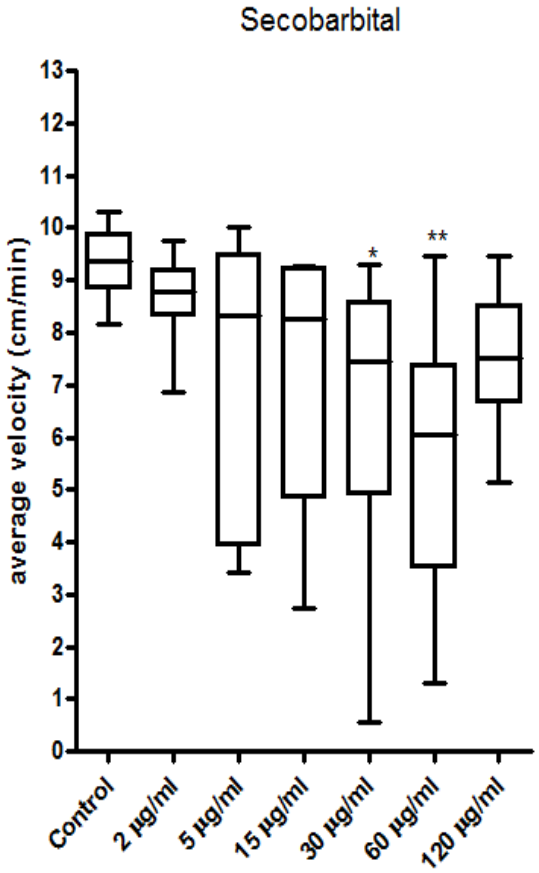

Figure 19 GraphPad Prism Result for Secobarbital

Table 6. *Behavioral count for Methamphetamine (MA) and Secobarbital (SB)*

Conc. / Behavior	2 µg/ml		5 µg/ml		15 µg/ml		30 µg/ml		60 µg/ml		120 µg/ml	
	MA	SB	MA	SB	MA	SB	MA	SB	MA	SB	MA	SB

HLC	0	1	0	2	0	2	2	4	2	3	2	0

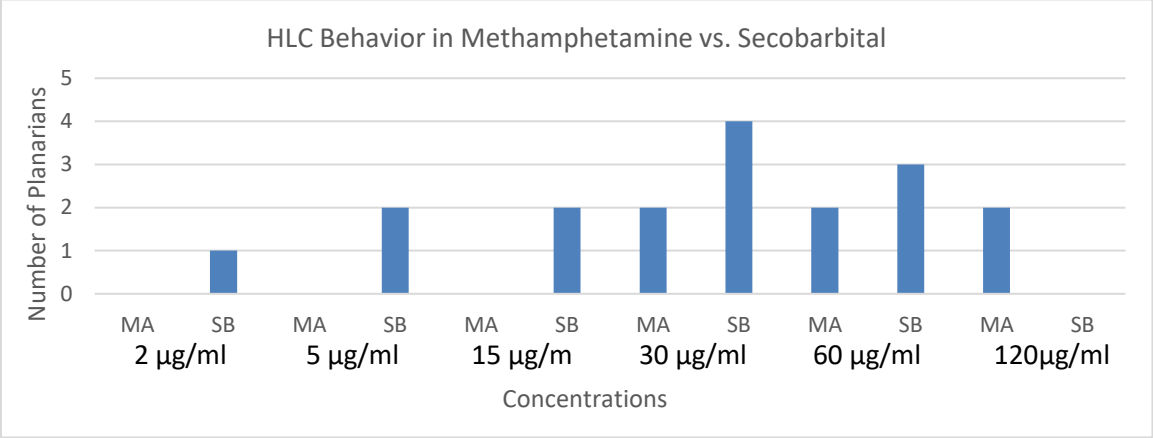

Figure 20 Plotted result of HLC behavior analysis

Cannabinoids

Delta-9-THC in 5 ml acetonitrile, MA-CHMINACA in 5 ml acetonitrile, and JWH 133 in 5 ml methyl acetate were successfully nitrogen evaporated and reconstituted by solvating in pure PEG400 to create dilutions for testing. Dissolution was assisted by sonication and gentle heating.

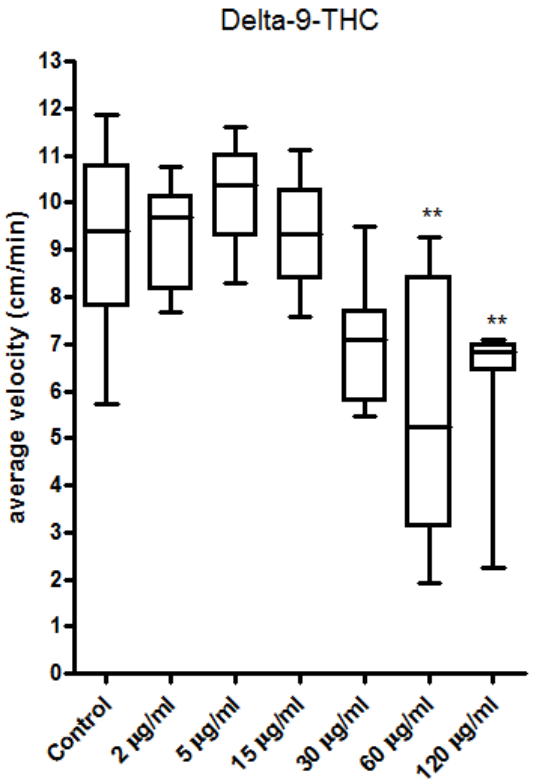

Figure 21 GraphPad Prism Result for Delta-9-THC

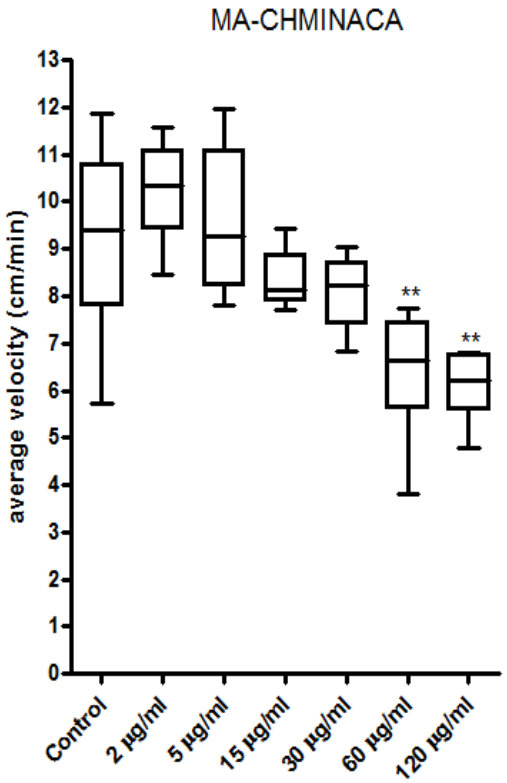

Figure 22 GraphPad Prism Result for MA-CHMINACA

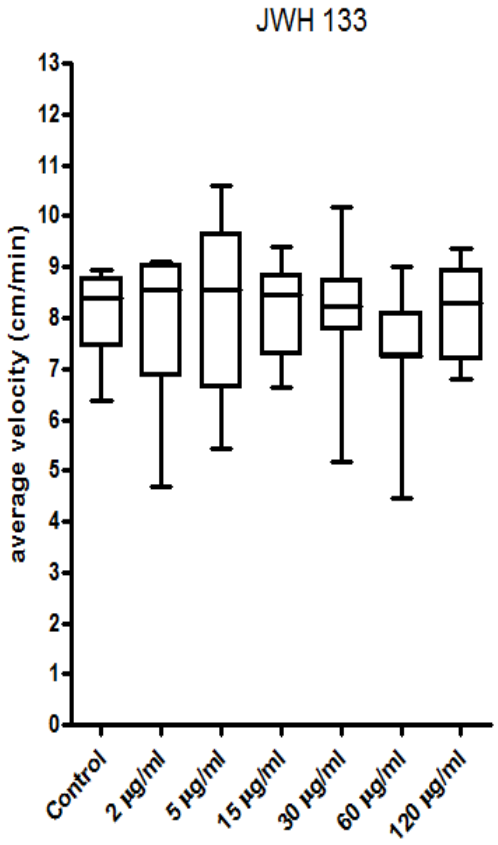

Figure 23 GraphPad Prism Result for JWH 133

Behavioral analysis was performed by observing the recorded videos. Both drugs (Figure 21 & 22) have shown to exhibit an abnormal behavior where planarians seemed to avoid the liquid surface and preferred the dry surface on the side of the petri dish. Planarians would proceed to Crawl Out of Water for Extended periods of time (COE) and performed either curling or hyperkinesia on the dry surface. Planarians that did not crawl out would exhibit some sudden head swings, however, was not in a sizeable number, and most have remained the same as normal behavior. Post-24-hour observation has shown that Delta-9-THC had a mortality rate higher than MA-CHMINACA at concentrations of 60 µg/ ml and 120 µg/ml showing all planarians have died in delta-9-THC while about two to four planarians survived in MA-CHMINACA. JWH 133 (Figure 23) has also not displayed any abnormal behavior and although some seem to display a sudden hyperkinesia but have returned to normal as recording time went on, unlike the other two cannabinoids. Post-24-hour observation showed normal activity with no mortality. Since JWH 133 did not display abnormal behavior, only Delta-9-THC and MA-CHMINACA behavior are tabulated base on how many drugs displayed COE in Table 7 and Figure 24.

Table 7. *Behavioral count for Delta-9-THC(D9) and MA-CHMINACA (MC)*

Conc. / Behavior	2 µg/ml		5 µg/ml		15 µg/ml		30 µg/ml		60 µg/ml		120 µg/ml	
	D9	MC	D9	MC	D9	MC	D9	MC	D9	MC	D9	MC
COE	1	0	3	0	3	6	6	7	6	8	8	8

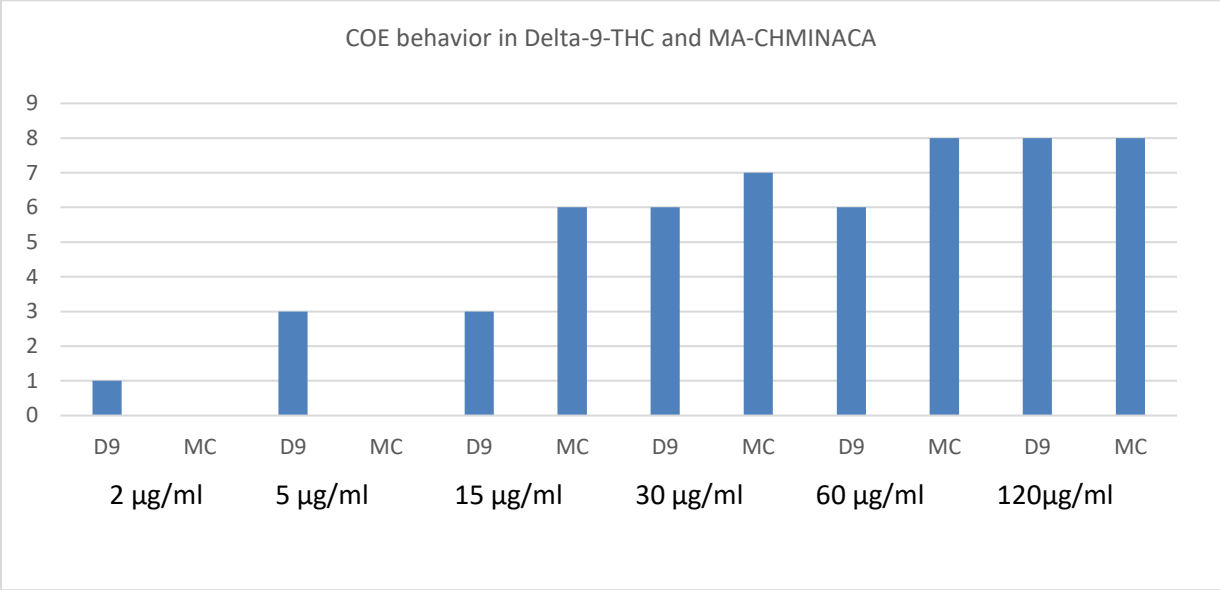

Figure 24 Plotted result of Cannabinoids behavior

Psychedelic

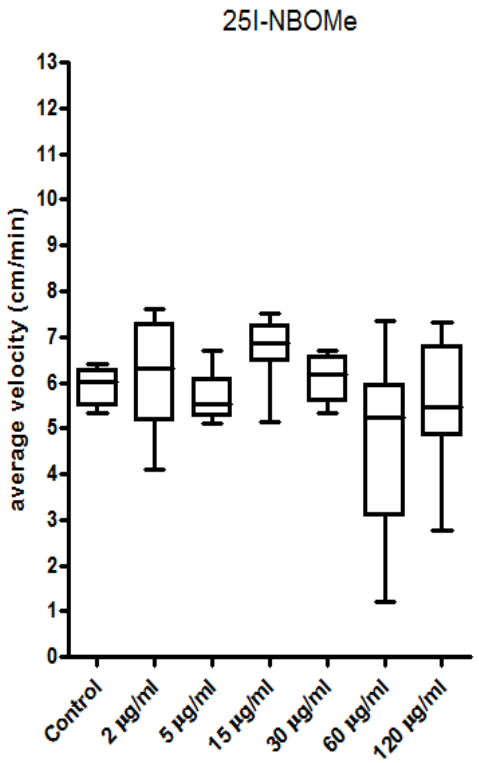

Figure 25 GraphPad Prism Result for 25I-NBOMe

25I-NBOMe in methanol was successfully nitrogen evaporated and reconstituted by solvating in pure PEG400 to create dilutions for testing. Dissolution was assisted by sonication and gentle heating.

Behavior analysis was done through observing the recorded videos. 25I-NBOMe (Figure 25) had shown to exhibit behavior in even the lowest concentration. Showing a characteristic

where planarians would experience a sudden hyperkinesia to the point of slowing their motility or stop in motion completely but a minute or few later would return to normal behavior. This behavior also varied at different concentration whereas the lower concentrations the planarians will experience a shorter duration of hyperkinesia and show no sign of motionless behavior, this was the opposite at higher concentration where planarians experienced a longer duration of hyperkinesia and result in motionless contraction before returning to normal. We will categorize the behavior in terms of how many planarians showed the Sudden Hyperkinesia (SHK) and the severity of stopping motionless then return to normal (SAG) in both Table 8 and Figure 26. Post-24-hour observation showed normal activity with no mortality.

Table 8. *Behavioral count for 25I-NBOMe*

Conc. / Behavior	2 µg/ml	5 µg/ml	15 µg/ml	30 µg/ml	60 µg/ml	120 µg/ml
SHK	5	6	7	8	8	8
SAG	0	0	0	5	5	8

Figure 26 Plotted result of 25I-NBOMe behavior

Cocaine

Cocaine in 5 ml hydrogen chloride was successfully nitrogen evaporated and reconstituted by solvating in pure PEG400 to create dilutions for testing. Dissolution was assisted by sonication and gentle heating.

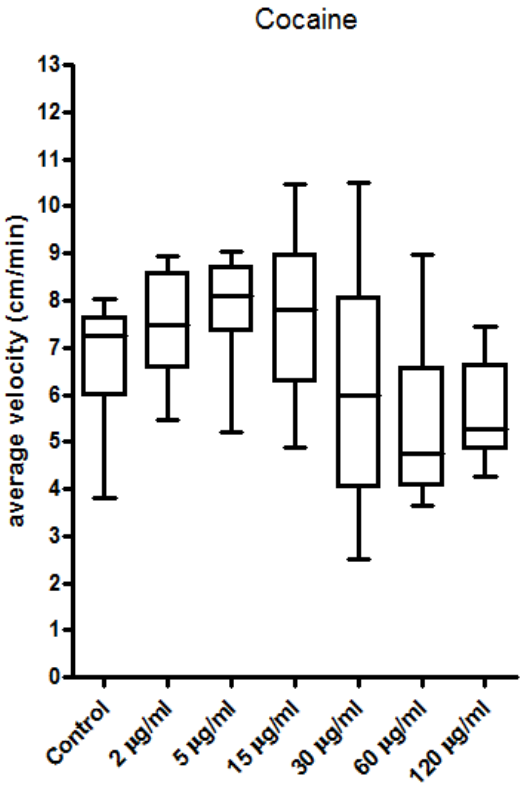

Figure 27 GraphPad Prism Result for Cocaine

Behavior analysis was done through observing the recorded videos. Cocaine's (Figure 27) behavioral observation was followed by literature review to account for C-Like Position (CLP), Screw-Like Hyperkinesia (SLH), and completely motionless. In our observation, abnormal behavior started to occur only around medium to the high concentration, and no Screw-like motion was observed. Post-24-hour observation showed normal activity with no mortality. Results will be tabulated (Table 9) but will not be graphed since there is not a significant amount of data for a graphical representation.

Table 9. *Behavioral count for Cocaine*

Conc. / Behavior	2 µg/ml	5 µg/ml	15 µg/ml	30 µg/ml	60 µg/ml	120 µg/ml
CLP	0	0	0	0	2	8
Motionless	0	0	0	1	1	2

CHAPTER 5

Discussion

General Observation

It was important to track the reproducibility of this method over time. As seen in in figures 8 & 9, most control runs (planarians in drug-free diluted salt solution only) preformed consistently well with more than one operator over multiple months and different batches of planarians. The control behavior was also very consistent in which the light-stimulated-planarians are shown to move in a circular motion and follow the outline of the petri dish with occasional directional change or crossing the middle of the dish. This general behavior of planarians allows us to compare various drug exposures performed.. OBS software (https://obsproject.com/) also has the ability to select videos recorded at different concentration to make a composite recording to show a comparison in real-time which greatly enhances the differences in behavior. Each observation was also run through VLC media player software (http://www.videolan.org) at different speeds to minimalize exaggeration by external factors and increase efficiency. Multi-approach to the behavioral analysis had helped us determine should the abnormal behavior arise whether it should be count toward our behavioral analysis data or disregard it as an artifact/noise from external factors. We believe our method provided us with great scrutiny to ensure the accuracy and precision.

Opioids

From the data, one can observe that locomotor activity generally decreases as planarians are exposed to opioids. In comparison, Furanyl Fentanyl had shown a dramatic decrease in velocity where its average velocity dipped to 4 or 5 cm.min?. In comparison

to morphine, Furanyl Fentanyl also observed to have significance differences at 15 μg/ml, 60 μg/ml, and 120 μg/ml using Dunnett's test while morphine has shown no significance difference from the control. We can further explain the behavioral patterns that were affecting the planarians' locomotor activity by looking at Table 10 below:

Table 10. *Locomotor Activity Affected by Behavioral Pattern for Opioids*

Drug Name & Concentration	Significant Locomotion	Behavior Phenotype (>50%)
Morphine at 2 μg/ml	No	None
Morphine at 5 μg/ml	No	None
Morphine at 15 μg/ml	No	SLM
Morphine at 30 μg/ml	No	SLM, SLH
Morphine at 60 μg/ml	No	SLM, SLH
Morphine at 120 μg/ml	No	SLM, SLH
F. Fentanyl at 2 μg/ml	No	SLM
F. Fentanyl at 5 μg/ml	No	SLM, SLH
F. Fentanyl at 15 μg/ml	Yes (*)	SLM, SLH
F. Fentanyl at 30 μg/ml	No	SLM, SLH, CLP
F. Fentanyl at 60 μg/ml	Yes (**)	SLM, SLH, CLP
F. Fentanyl at 120 μg/ml	Yes (*)	SLM, SLH, CLP

Behavior analysis closely resembled reports by Passarelli's Opioid–dopamine interaction in planaria. In this behavioral study [9], multiple behavorl motions were observed that include conrrol conditionas and circulating the dish and wide turns; snake-like motion (SLM) where planarians perform an S-shape motion or snaking out of the dish; screw-like hyperkinesia (SLH) showing extreme twisting on multiple occasions, closely resemble a screw, and C-Like position (CLP) that showed planarians forming a C-shape and curling up. Passarelli's observation saw an increased motor activity with slight CLP and SLH response after exposure to low doses and at higher doses caused the enhancement of SLH and CLP activities, together with dose-related SLM activity [9]. Our observation, however, slightly deviated from this observation. At lower concentrations both SLM and SLH were observed and CLP behavior was much more

distinguishable at a higher concentration. We believed that CLP causes a drop in velocity as evident in the video recording that showed planarians displaying the CLP would curl up and maintain the C-like but motionless thus no longer expressing SLM or SLH. Post-24 hour observation had also shown that planarians are forming CLP generally were not moving and remained in CLP motionlessly. Our explanation on such behavioral progression is also dosage and potency dependent as one can observe the progression from morphine was much slower while the much more potent Furanyl Fentanyl can result in CLP of a couple of planarians within 5 μg/ml. Therefore, CLP was a good indicator of dosage response for our experiment since in a higher concentration such as 60 μg/ml and 120 μg/ml where CLPs were expressed very early in the videos causing these group of concentrations to show much lower velocity than previous concentrations. At 30 μg/ml, however, the portion of planarians expressing CLP was not observed until a later stage of the video and was actively expressing SLM and SLH before showing CLP behavior. Short-term-hyperkinesia in some of the planarians can also explain the lower bracket of the data. However, such behavior was not prominent in our experiment as previously reported by literature.

Amphetamines

From the data, both stereoisomers of amphetamines showed a general increase in locomotion of planarians thus confirming the previous literature where planarian's locomotor activity increased after exposure to amphetamine as a stimulant [19]. However, R(-)-amphetamine, the levorotation enantiomer of the amphetamine molecule has shown to affect locomotion throughout concentration in a much more pronounced and dramatic increase or decrease than S(+)-amphetamine. Further statistical analysis has

also shown a significant difference at 60 μg/ml for R(-)-amphetamine in comparison with control using the Dunnett's test while S(+)-amphetamine has no significance difference in any of the concentrations. We can further explain the behavioral patterns that were affecting the planarians' locomotor activity by looking at Table 11 below:

Table 11. *Locomotor Activity Affected by Behavioral Pattern for Amphetamines*

Drug Name & Concentration	Significant Locomotion	Behavior Phenotype (>50%)
S(+)-amphetamine at 2 μg/ml	No	EHS, HTM
S(+)-amphetamine at 5 μg/ml	No	EHS, HTM
S(+)-amphetamine at 15 μg/ml	No	EHS, CST, HTM
S(+)-amphetamine at 30 μg/ml	No	EHS, CST, HTM
S(+)-amphetamine at 60 μg/ml	No	EHS, CST, HTM
S(+)-amphetamine at 120 μg/ml	No	EHS, CST, HTM
R(-)-amphetamine at 2 μg/ml	No	EHS, CST, HTM
R(-)-amphetamine at 5 μg/ml	No	EHS, CST, HTM
R(-)-amphetamine at 15 μg/ml	No	EHS, CST, HTM
R(-)-amphetamine at 30 μg/ml	No	None
R(-)-amphetamine at 60 μg/ml	Yes (*)	CC

Previous clinical literature has reported that S(+)-amphetamine is generally more potent than R(-)-amphetamine in human [18] thus conflicting our expectation that S(+)-amphetamine should've shown more dramatic effects on locomotor activity. Nevertheless, planarians as an invertebrate animal have shown the opposite effects which are of significant interest for further research in receptor activity within the neural cluster. This difference in expectation of the outcome of the locomotor activity will be noted, and interpretation of the reaction upon different stereoisomer should be analyzed along with behavioral profiles to confirm the differences.

Behavioral analysis showed many patterns with the most prominent behavioral pattern for both stereoisomers were Exaggerated Head-swings (EHS) and Head-twitch movement (HTM). Although these two behaviors are not unique to amphetamine, they are widely observable in almost all planarians throughout all concentrations. EHS and

HTM behaviors can likely explain the reason for the increase in locomotion due to the dramatic movement prompted by these behaviors causing the tracking system to pick up on the excess movement. C-shaped Twisting (CST) behavior was secondary at the start of S(+)-amphetamine and gradually increase in observation in following concentrations, this can explain why R(-)-amphetamine had a generally higher locomotor activity in lower concentrations due to its CST behavior was much more projecting and consistent than S(+)-amphetamine thus more excessive movement was picked up by the tracking system. At concentration 30 μg/ml and 60 μg/ml of R(-)-amphetamine, planarians start to exhibit Curling Contraction (CC) behavior which caused a dramatic decrease in displaying other behaviors due to most of them remained motionless, which also explained the sudden drop in locomotor activity. S(+)-amphetamine exposed planarians did not display any CC behavior thus their locomotor activity was not significantly affected. Lastly, many of the planarians exposed to S(+)-amphetamine throughout the concentrations crawled out of the petri dish but returned shortly after. The crawling out behavior likely cause S(+)-amphetamine exposed planarians not to reach a higher locomotor activity when compared to R(-)-amphetamine due to the short pause in activity for being out of the petri dish. Retesting for R(-)-amphetamine is recommended for the future drug exposure experiment. Due to unforeseen circumstances, there were not enough planarians for testing of the 120 μg/ml which make the data set incomplete, however, from observation, we would predict the higher concentration would show more planarians experience CC behavior thus result in a lower locomotor activity and a greater decrease in velocity.

Benzodiazepines and Nonbenzodiazepines

Planarians with Zolpidem exposure showed a significant decrease in locomotion with concentrations at 60 μg/ml and 120 μg/ml showed a significantly different from control by the Dunnett's test. As a Z-class drug, Zolpidem's effect on planarian's locomotion was expected as nonbenzodiazepine class drugs often act like benzodiazepines to have a sedative and hypnotic-like effect. The behavioral patterns that were affecting the planarians' locomotor activity will be expressed by Table 12 below:

Table 12. *Locomotor Activity Affected by Behavioral Pattern for Zolpidem*

Drug Name & Concentration	Significant Locomotion	Behavior Phenotype (>50%)
Zolpidem at 2 μg/ml	No	None
Zolpidem at 5 μg/ml	No	CSC
Zolpidem at 15 μg/ml	No	CSC
Zolpidem at 30 μg/ml	No	CSC, TAC
Zolpidem at 60 μg/ml	Yes (*)	CSC, TAC, EAC
Zolpidem at 120 μg/ml	Yes (**)	CSC, TAC, EAC

Zolpidem has elicited unique behavioral pattern from planarians that were not seen in other drugs. Starting with initial C-shape curling (CSC) where, unlike previous C-like position, planarians will continue to move in a small circular twisting pattern without stopping. CSC can likely explain the drop in locomotor activity since when compare to the normal behavior, CSC decreased in circumference, and thus planarians traveled area was decreased. Around medium concentration, behavioral pattern progressed into a twitching and curling in place behavior (TAC) in which the planarians would display exaggerated twitching, and curling movement of both their tail and head but remain in the same location. This behavior will, although not necessarily decrease planarians' activity, have a dramatic effect on the tracking system since planarians are displaying this behavior in place; distance recorded will decrease significantly resulting in a lower velocity. Lastly at higher concentrations, behavior pattern known as Extending and

Contracting in place (EAC) had shown planarians to be remaining in place while displaying an extension of its body dorsally and contracting right after the extension. This behavior was much less dramatic than TAC since our tracking method is not sensitive enough to track such minuscule behavior, but it is observable with our naked eyes. EAC behavior was a very good indicator of the presence of high concentration of Zolpidem allowing us to construct a dosage response curve accordingly.

Barbiturate vs. Methamphetamine

Since this drug exposure experiment of Barbiturate vs. Methamphetamine was only concerned with the lack of locomotor activity known as hyperkinesia-like C-shape curl (HLC) threshold for behavioral pattern observed will be lower than previous standards. Table 13 below will be used for further discussion of the characteristics we saw.

Table 13. *Locomotor Activity Affected by Behavioral Pattern for HLC*

Drug Name & Concentration	Significant Locomotion	Behavior Phenotype (>1 Sample)
Methamphetamine at 2 μg/ml	No	None
Methamphetamine at 5 μg/ml	No	None
Methamphetamine at 15 μg/ml	No	None
Methamphetamine at 30 μg/ml	No	2 HLC
Methamphetamine at 60 μg/ml	No	2 HLC
Methamphetamine at 120 μg/ml	No	2 HLC
Secobarbital at 2 μg/ml	No	None
Secobarbital at 5 μg/ml	No	2 HLC
Secobarbital at 15 μg/ml	No	2 HLC
Secobarbital at 30 μg/ml	Yes (*)	4 HLC
Secobarbital at 60 μg/ml	Yes (**)	3 HLC
Secobarbital at 120 μg/ml	No	None

Our expectation for methamphetamine was for it to show a dramatic increase in velocity, however, likely due to the potency of methamphetamine we saw amphetamine-like behavior early in the lower dosage including EHS, CST, and HTM which likely explained the higher locomotor activity when compared to Secobarbital.

Methamphetamine behavioral pattern then slowly progress into a motionless behavior (known in this case HLC) very rapidly. Secobarbital displayed some similar characteristics including EHS and HTM, however, was less dramatic and statistically insignificant (about one to two planarians) yet at lower dosage Secobarbital exposed planarians were already displayed HLC behavior at a much more aggressive pace. This result can explain why Secobarbital saw a much more significant drop in locomotor activity. In comparison, at a lower dosage, the two drugs did show a difference in locomotor activity allowing us to distinguish between stimulant and depressant. Nevertheless, the reason for the same behavioral display at a higher dosage of both drugs can possibly be related to the toxicity of the substance. Both substances are known to have high potency and toxicity with very narrow median lethal dosage (LD_{50})[17] which likely contribute to the planarians displayed of acute toxicity. This allows us to label HLC as a good general indicator of substance with high toxicity level.

Cannabinoids

The intent and purpose of the cannabinoids' experiment were designed to see if planarians can distinguish between different cannabinoid receptor binding. Tetrahydrocannabinol (Delta-9-THC) is the principle psychoactive substance of cannabis that is a partial agonist of both CB_1 and CB_2 receptor [31]. We decided to use Tetrahydrocannabinol as a baseline to examine other cannabinoids with different affinity for either CB_1 or CB_2. Synthetic cannabinoids, MA-CHMINACA with higher affinity for the CB_1 receptor and JWH 133 with higher affinity for CB_2, were chosen to examine and compared to Tetrahydrocannabinol. From a quick glance, graphical data from both delta-9-THC and MA-CHMINACA showed a sudden increase in locomotor activity in early

concentrations and slowly show signs of hypomotility as concentrations increased while JWH 133 remained very consistent in terms of locomotor activity despite the differences in concentrations. Planarians affected by the CB_1 receptor agonist would proceed to crawl out of water for extended periods of time (COE) and performed either curling or hyperkinesia on the dry surface. COE can be account for the loss of velocity in locomotor activity due to the system tracker not able to pick up z-axis movement out of the petri dish. This abnormal movement resulted in a significant increase in pixel density at a specific spot, however, not the pixel distance thus program calculation of distance traveled was decreased. Likewise, the hyperkinesia and curling motion while out of the water surface results in a sudden stop or motionless movement for a period of time which can also account the loss of distance traveled. This behavior was not observed previously in other drug exposure and is unique for CB_1 receptor agonist. The behavioral patterns that were affecting the planarians' locomotor activity will be expressed by the Table 14 below for all of the cannabinoids tested:

Table 14. *Locomotor Activity Affected by Behavioral Pattern for Cannabinoids*

Drug Name & Concentration	Significant Locomotion	Behavior Phenotype (>50%)
Delta-9-THC at 2 μg/ml	No	None
Delta-9-THC at 5 μg/ml	No	None
Delta-9-THC at 15 μg/ml	No	None
Delta-9-THC at 30 μg/ml	No	6 COE
Delta-9-THC at 60 μg/ml	Yes (*)	6 COE
Delta-9-THC at 120 μg/ml	Yes (**)	8 COE
MA-CHMINACA at 2 μg/ml	No	None
MA-CHMINACA at 5 μg/ml	No	None
MA-CHMINACA at 15 μg/ml	No	6 COE
MA-CHMINACA at 30 μg/ml	No	7 COE
MA-CHMINACA at 60 μg/ml	Yes (*)	8 COE
MA-CHMINACA at 120 μg/ml	Yes (**)	8 COE
JWH 133 at 2 μg/ml	No	None
JWH 133 at 5 μg/ml	No	None

JWH 133 at 15 μg/ml	No	None
JWH 133 at 30 μg/ml	No	None
JWH 133 at 60 μg/ml	No	None
JWH 133 at 120 μg/ml	No	None

By comparison, we can see that CB_1 receptor agonist, MA-CHMINACA resembles closely to Tetrahydrocannabinol in terms of behavioral effects and significant locomotion concentrations while CB_2 receptor agonist, JWH 133, showed no sign of behavioral pattern displayed by the previous two drugs. In fact, after observation of JWH 133, revealed that no significant abnormal behavior was observable from JWH 133 and planarians that were exposed continued to display normal baseline behavior. This result indicates that planarians are able to differentiate drugs with different receptor affinity regardless of structure similarity between the molecules and can likely explain the possible existence of cannabinoid receptors in the neural cluster as reported in previous literature [32].

Psychedelics

25I-NBOMe was tested and have shown unique behavior from observation despite the lack of significant locomotor activity. Nevertheless, behavioral patterns were observed that enable us to construct a possible dosage response. One of the most prominent behaviors described from the result section is Sudden Hyperkinesia (SHK). This behavior has shown planarians to perform a normal behavior and a sudden twitching in motion for a few minutes before returning to normal behavior again. If a sudden motionless stop was performed the behavior know as Stop and Go (SAG) was also noted and recorded. Behavior pattern also varied at different concentration whereas the lower concentrations the planarians will experience a shorter duration of SHK and show no sign of SAG behavior, this was the opposite at higher concentration where planarians

experienced a longer duration of SHK and result in SAG behavior before returning to normal. The behavioral patterns will be expressed by the Table 15 below:

Table 15. *Locomotor Activity Affected by Behavioral Pattern for 25I-NBOMe*

Drug Name & Concentration	Significant Locomotion	Behavior Phenotype (>50%)
25I-NBOMe at 2 μg/ml	No	SHK
25I-NBOMe at 5 μg/ml	No	SHK
25I-NBOMe at 15 μg/ml	No	SHK
25I-NBOMe at 30 μg/ml	No	SHK, SAG
25I-NBOMe at 60 μg/ml	No	SHK, SAG
25I-NBOMe at 120 μg/ml	No	SHK, SAG

Locomotor activity mean values between all concentrations are fairly close and only show a slight decrease in higher concentration. The relationship between behavioral pattern affecting locomotor activity can be explained by the observation that all planarians that elicit SHK and SAG contraction all returned to normal behavior after a period of time and even some planarians have observed to increase in activity after experiencing SHK or SAG, thus explain the likelihood of no significance in locomotor activity.

Cocaine

Cocaine was tested in part to confirm previous literature report of an increase in locomotor activity in planarians when exposed to cocaine [41]. Cocaine's behavioral pattern was well documented by Palladini's paper: A Pharmacological Study of Cocaine Activity in Planaria. The author of the article has shown a total of two different observable behavior including motionless and C-like position (CLP) at increasing concentrations of cocaine or similar receptor agonists. Due to the nature of our experiment design and following the standard of procedures our concentrations were tested at a different level when compared to the previous reports. The behavioral patterns

that we observed that were affecting the planarians' locomotor activity will be expressed by Table 16 below:

Table 16. *Locomotor Activity Affected by Behavioral Pattern for Cocaine*

Drug Name & Concentration	Significant Locomotion	Behavior Phenotype (>50%)
Cocaine at 2 μg/ml	No	None
Cocaine at 5 μg/ml	No	None
Cocaine at 15 μg/ml	No	None
Cocaine at 30 μg/ml	No	None
Cocaine at 60 μg/ml	No	None
Cocaine at 120 μg/ml	No	CLP

From literature report, cocaine expressed motionless at 20 μg/ml concentration and prominent CLP behavior at 100 μg/ml. The behavior observed at an approximate concentration such as 30 μg/ml and 100 μg/ml did show planarians display behaviors such as motionless (at a total count of 4) and CLP (at a total count of 10) respectively. This experiment on cocaine was another good indication of reproducibility regarding drugs that were tested.

Overall Data Interpretation

Table. 17 *Overall data tabulation*

Stimulants	**Concentrations**	**Locomotion Significance**	**Behavior Phenotype (>50%)**	**Post-24 Hour Mortality (>50%)**
S(+)-Amphetamine	60 µg/ml	No	EHS, CST, HTM	No
	120 µg/ml	No	EHS, CST, HTM	No
R(+)-Amphetamine	60 µg/ml	No	None	No
	120 µg/ml	Yes (*)	CC	No
Methamphetamine	60 µg/ml	No	None	Yes
	120 µg/ml	No	None	Yes
Cocaine	60 µg/ml	No	None	No
	120 µg/ml	No	CLP	No
Depressants	**Concentrations**	**Locomotion Significance**	**Behavior Phenotype (>50%)**	**Post-24 Hour Mortality (>50%)**
Zolpidem	60 µg/ml	Yes (*)	CSC, TAC, EAC	No
	120 µg/ml	Yes (**)	CSC, TAC, EAC	No
Secobarbital	60 µg/ml	Yes (**)	None	Yes
	120 µg/ml	No	None	Yes
Cannabinoids	**Concentrations**	**Locomotion Significance**	**Behavior Phenotype (>50%)**	**Post-24 Hour Mortality (>50%)**
Delta-9-THC	60 µg/ml	Yes (*)	COE	Yes
	120 µg/ml	Yes (**)	COE	Yes
MA-CHMINACA	60 µg/ml	Yes (*)	COE	No
	120 µg/ml	Yes (**)	COE	No
JWH 133	60 µg/ml	No	None	No
	120 µg/ml	No	None	No
Opioids	**Concentrations**	**Locomotion Significance**	**Behavior Phenotype (>50%)**	**Post-24 Hour Mortality (>50%)**
Morphine	60 µg/ml	No	SLM, SLH	No
	120 µg/ml	No	SLM, SLH	Yes
Furanyl Fentanyl	60 µg/ml	Yes (**)	SLM, SLH, CLP	Yes
	120 µg/ml	Yes (*)	SLM, SLH, CLP	Yes
Psychedelic	**Concentrations**	**Locomotion Significance**	**Behavior Phenotype (>50%)**	**Post-24 Hour Mortality (>50%)**
25I-NBOMe	60 µg/ml	No	SHK, SAG	No
	120 µg/ml	No	SHK, SAG	No

EHS = Exaggerated Head Swing, **CST** = C-shape twisting, **HTM** = Head-twitch movement, **CC** = Curling Contraction, **CLP** = C-like position, **CSC**= C-shape curling, **TAC** = twitching and curling in place, **EAC** = Extending and Contracting in place, **COE** = crawl out of water for extended time, **SLM** = Snake-like movement, **SLH** = Screw-like hyperkinesia, **SHK** = sudden hyperkinesia, **SAG** = Stop and go motion

From the overall tabulated data in Table 17, one can observe several interesting results from this study. For example, our results suggest the ability of the model to distinguish enantiomers of the stimulant class drug amphetamine. With prototypical greater behavior displayed with S(+)-Amphetamine compared to R(+)-Amphetamine,

where there were no observable behaviors coupled with decreased locomotor activity. For the cannabinoids compounds we observed that compounds with moderate to strong CB_1 agonist activity, but not CB2 agonist activity, have resulted in defined behavio of crawling out of the test soluteion (COE), and locomotor activity decreases. Lastly, although psychedelic class has shown no significance in locomotor activity, 25I-NBOMe had a quantifiable behavioral phenotype, we called SKG and SAG that was unique to this class of drug and demonstrated good dose-response.

Conclusion

The planarian animal model system has proved itself to be both cost and time efficient tool that has not only great utility in an academic laboratory setting but also has the potential to study the pharmacological profile of drugs further. This model system is easy to maintain and boast great reproducibility between multiple operators. The cultivation, feeding, and measuring of locomotor activity require minimal capital and consumable costs making it desirable as well as competitive to some other animal models. As demonstrated in results, this method has achieved good reproducibility over a long period of time and the ability to study the adverse behavioral effects of illicit and prescription drugs from the discussion. Our method further achieved a semi-automated operation utilizing freely available software to develop automation macros and increased efficiency through the multi-channeled system. Nevertheless, such method was comparable to similar methods used in the literature. Using a suitable drug solvents (PEG400) in this system, we were able to observe behavioral changes in a side by side comparison to distinguish abnormal behavior and derive from such quantification to discuss possible receptor affinity dependency changes affecting planarian locomotor

activity. The results further suggest the model can distinguish between differences affinity within different neural receptors preferring compounds in vertebrate; making the model potentially useful diagnostic tool for forensic toxicologists and legislators to learn more about the adverse effect profile of drugs and other toxins.

Future Prospect

Future studies may expand this query to determine the effect of sub-chronic and chronic drug exposures; alternate Dugesia species, co-solvent systems, and other synthetic drugs. Most importantly efficiency can be further improved with sophisticated robotic augments much like the current enzyme-linked immunosorbent assay system using robotic pipetting and washer. Imaging software can also use further improvement to obtain improved resolution and allows three-dimensional image capturing. Captured images and videos can also use more sophisticated software to measure turn angles or cumulative time data to improve both statistical and behavioral analysis by providing more in-depth data. The same thing can also be done to the biological aspect of the planarian animal model where further genomic sequencing and histology will provide us with more information of neural receptor expression that exist within the animals and how it is affecting their locomotion or behavior.

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APPENDIX

A .Calculations

1. Drug concentration (assuming 10 mg/ml stock)

   Make a 20 ml stock solution for n=8 animals per concentration

   - 2 µg/ml

     (Stock) (Volume needed) = (Conc. wanted) (Salt Solution)

     Volume needed = (2 µg/ml) (20 ml) / (10,000 µg/ml) = 0.004 ml = 4 µL

   - 5 µg/ml

     (Stock) (Volume needed) = (Conc. wanted) (Salt Solution)

     Volume needed = (5 µg/ml) (20 ml) / (10,000 µg/ml) = 0.01 ml = 10 µL

   - 15 µg/ml

     (Stock) (Volume needed) = (Conc. wanted) (Salt Solution)

     Volume needed = (15 µg/ml) (20 ml) / (10,000 µg/ml) = 0.03 ml = 30 µL

   - 30 µg/ml

     (Stock) (Volume needed) = (Conc. wanted) (Salt Solution)

     Volume needed = (30 µg/ml) (20 ml) / (10,000 µg/ml) = 0.06 ml = 60 µL

   - 60 µg/ml

     (Stock) (Volume needed) = (Conc. wanted) (Salt Solution)

     Volume needed = (60 µg/ml) (20 ml) / (10,000 µg/ml) = 0.12 ml = 120 µL

   - 120 µg/ml

     (Stock) (Volume needed) = (Conc. wanted) (Salt Solution)

     Volume needed = (120 µg/ml) (20 ml) / (10,000 µg/ml) = 0.24 ml = 240 µL

2. Pixel to distance conversion

Using ImageJ Linear tool, we are able to measure pixels exist on image plane and approximate camera distance to recorded image. The amount of pixels in the image was measured to be 37 pixels per centimeter in length thus:

- Distance Travel = Length (pixels/cm) / 37 pixels
- Average Velocity = Total Length (cm) / 5 mins

B. Tables for Raw Data

1. Opioids

Table 1 *Control group testing for Opioids*

Control (pixels)	Distance (cm)	Velocity (cm/min)
1463.318	39.549	7.910
1001.858	27.077	5.415
1721.896	46.538	9.308
1351.812	36.535	7.307
1457.399	39.389	7.878
1901.502	51.392	10.278
1195.791	32.319	6.464
1300.482	35.148	7.030
	Average	7.699

Table 2 *Locomotion Results of Morphine*

2 μg/ml	Dis. (cm)	V(cm/min)	5 μg/ml	Dis. (cm)	V(cm/min)	15 μg/ml	Dis. (cm)	V(cm/min)
1897.478	51.283	10.257	1277.331	34.522	6.904	1377.542	37.231	7.446
906.122	24.490	4.898	1163.249	31.439	6.288	2070.887	55.970	11.194
1715.215	46.357	9.271	1663.724	44.966	8.993	1716.739	46.398	9.280
2170.316	58.657	11.731	1941.551	52.474	10.495	1345.558	36.366	7.273
1297.129	35.058	7.012	1221.016	33.000	6.600	1997.458	53.985	10.797
789.037	21.325	4.265	1176.868	31.807	6.361	125.141	3.382	0.676
1187.556	32.096	6.419	1082.406	29.254	5.851	806.512	21.798	4.360
2177.531	58.852	11.770	1873.101	50.624	10.125	1957.186	52.897	10.579
	Average	8.203		Average	7.702		Average	7.701
30 μg/ml	Dis. (cm)	V(cm/min)	60 μg/ml	Dis. (cm)	V(cm/min)	120μg/ml	Dis. (cm)	V(cm/min)
1843.003	49.811	9.962	1713.886	46.321	9.264	1077.366	29.118	5.824
1132.688	30.613	6.123	1972.408	53.308	10.662	1968.901	53.214	10.643
1762.325	47.630	9.526	1594.131	43.085	8.617	591.086	15.975	3.195
2184.338	59.036	11.807	564.073	15.245	3.049	1241.767	33.561	6.712
1844.746	49.858	9.972	1232.458	33.310	6.662	1333.061	36.029	7.206
1578.827	42.671	8.534	1776.648	48.018	9.604	1390.630	37.585	7.517
2024.487	54.716	10.943	1144.161	30.923	6.185	1330.973	35.972	7.194
1311.017	35.433	7.087	1499.305	40.522	8.104	1406.748	38.020	7.604
	Average	9.244		Average	7.768		Average	6.987

Table 3 *Locomotion Results of Furanyl Fentanyl*

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
1228.268	33.196	6.639	363.893	9.835	1.967	931.452	25.174	5.035
1537.279	41.548	8.310	210.716	5.695	1.139	169.745	4.588	0.918
1860.089	50.273	10.055	1340.209	36.222	7.244	1145.947	30.972	6.194
1321.926	35.728	7.146	1356.864	36.672	7.334	1004.765	27.156	5.431
1556.186	42.059	8.412	874.499	23.635	4.727	994.998	26.892	5.378
877.082	23.705	4.741	1184.489	32.013	6.403	734.054	19.839	3.968
1804.751	48.777	9.755	1266.146	34.220	6.844	1005.363	27.172	5.434
117.835	3.185	0.637	1135.041	30.677	6.135	1448.803	39.157	7.831
	Average	6.962		Average	5.224		Average	5.024
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)	120 µg/ml	Dis. (cm)	V(cm/min)
938.991	25.378	5.076	777.184	21.005	4.201	793.004	21.433	4.287
1054.160	28.491	5.698	956.650	25.855	5.171	952.628	25.747	5.149
1434.063	38.758	7.752	234.512	6.338	1.268	896.699	24.235	4.847
861.613	23.287	4.657	1109.004	29.973	5.995	653.583	17.664	3.533
908.904	24.565	4.913	576.095	15.570	3.114	1019.375	27.551	5.510
1018.371	27.524	5.505	1021.461	27.607	5.521	776.963	20.999	4.200
1135.326	30.684	6.137	1111.605	30.043	6.009	773.937	20.917	4.183
1077.791	29.129	5.826	714.999	19.324	3.865	1012.842	27.374	5.475
	Average	5.695		Average	4.393		Average	4.648

Table 4 *Statistical analysis of Morphine*

Parameter	Value			
Table Analyzed				
Morphine				
One-way analysis of variance				
P value	0.6964			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	7			
F	0.6416			
R squared	0.07284			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	7.371			
P value	0.2879			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	df	MS	
Treatment (between columns)	22.95	6	3.826	
Residual (within columns)	292.2	49	5.963	
Total	315.1	55		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	-0.5041	0.4129	P > 0.05	-3.754 to 2.746
Control vs 5 mg/ml	-0.003375	0.002764	P > 0.05	-3.254 to 3.247
Control vs 15 mg/ml	-0.001875	0.001536	P > 0.05	-3.252 to 3.248

Control vs 30 mg/ml	-1.546	1.266	P > 0.05	-4.796 to 1.705
Control vs 60 mg/ml	-0.06962	0.05703	P > 0.05	-3.320 to 3.181
Control vs 120 mg/ml	0.7119	0.5831	P > 0.05	-2.538 to 3.962

Table 5 *Statistical analysis of Furanyl Fentanyl*

Parameter	Value			
Table Analyzed				
Furanyl Fentanyl				
One-way analysis of variance				
P value	0.0085			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	7			
F	3.286			
R squared	0.2869			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	17.52			
P value	0.0075			
P value summary	**			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	72.62	6	12.1	
Residual (within columns)	180.5	49	3.683	
Total	253.1	55		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	0.7369	0.768	P > 0.05	-1.817 to 3.291
Control vs 5 mg/ml	2.475	2.579	P > 0.05	-0.07965 to 5.029
Control vs 15 mg/ml	2.675	2.788	P < 0.05	0.1209 to 5.229
Control vs 30 mg/ml	2.003	2.088	P > 0.05	-0.5510 to 4.558
Control vs 60 mg/ml	3.306	3.445	P < 0.01	0.7515 to 5.860
Control vs 120 mg/ml	3.051	3.179	P < 0.05	0.4965 to 5.605

2. Amphetamine

Table 6 *Control group testing for Amphetamines*

Control (pixels)	Distance (cm)	Velocity (cm/min)
798.426	21.579	4.316
1523.143	41.166	8.233
1864.847	50.401	10.080
1562.742	42.236	8.447
1829.076	49.434	9.887
1419.953	38.377	7.675
1963.782	53.075	10.615
1376.966	37.215	7.443
	Average	8.337

Table 7 *Locomotion Results of R(-)-amphetamine and Amphetamines group control*

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
1736.988	46.946	9.389	904.581	24.448	4.890	1672.986	45.216	9.043
981.056	26.515	5.303	1762.437	47.633	9.527	1288.979	34.837	6.967
2123.186	57.383	11.477	1176.484	31.797	6.359	2237.266	60.467	12.093
1223.003	33.054	6.611	1491.877	40.321	8.064	2089.159	56.464	11.293
2334.771	63.102	12.620	2448.463	66.175	13.235	1763.073	47.651	9.530
1636.443	44.228	8.846	2608.831	70.509	14.102	1340.906	36.241	7.248
1749.631	47.287	9.457	2137.115	57.760	11.552	2403.255	64.953	12.991
2147.321	58.036	11.607	1879.549	50.799	10.160	1214.929	32.836	6.567
	Average	9.414		Average	9.736		Average	9.467
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)			
1254.570	33.907	6.781	462.654	12.504	2.501			
1805.891	48.808	9.762	475.040	12.839	2.568			
838.669	22.667	4.533	1434.241	38.763	7.753			
1316.014	35.568	7.114	1171.142	31.652	6.330			
1487.895	40.213	8.043	867.980	23.459	4.692			
1016.726	27.479	5.496	129.394	3.497	0.699			
518.826	14.022	2.804	1216.164	32.869	6.574			
1126.569	30.448	6.090	695.519	18.798	3.760			
	Average	6.328		Average	4.360			

Table 8 *Locomotion Results of S(+)-amphetamine*

2 µg/ml	Dis. (cm)	V(cm/min)	*5* µg/ml	Dis. (cm)	V(cm/min)	*15* µg/ml	Dis. (cm)	V(cm/min)
1889.703	51.073	10.215	1481.110	40.030	8.006	1395.340	37.712	7.542
1554.008	42.000	8.400	1184.654	32.018	6.404	1528.012	41.298	8.260
1198.958	32.404	6.481	1734.389	46.875	9.375	1816.055	49.083	9.817
857.504	23.176	4.635	2255.888	60.970	12.194	1372.445	37.093	7.419
2103.476	56.851	11.370	1962.389	53.038	10.608	1318.468	35.634	7.127
1623.894	43.889	8.778	1143.547	30.907	6.181	1494.413	40.390	8.078
2154.656	58.234	11.647	2151.355	58.145	11.629	1070.025	28.920	5.784
1962.958	53.053	10.611	1402.331	37.901	7.580	1084.890	29.321	5.864
	Average	9.017		Average	8.997		Average	7.486
30 µg/ml	Dis. (cm)	V(cm/min)	*60* µg/ml	Dis. (cm)	V(cm/min)	*120*µg/ml	Dis. (cm)	V(cm/min)
1396.057	37.731	7.546	1363.518	36.852	7.370	1138.603	30.773	6.155
1852.001	50.054	10.011	1711.188	46.248	9.250	1667.438	45.066	9.013
1378.772	37.264	7.453	1418.534	38.339	7.668	1308.335	35.360	7.072
1305.995	35.297	7.059	966.676	26.126	5.225	2115.755	57.183	11.437
1791.948	48.431	9.686	958.117	25.895	5.179	1348.037	36.433	7.287
2209.312	59.711	11.942	1374.892	37.159	7.432	1101.292	29.765	5.953
1339.032	36.190	7.238	1733.863	46.861	9.372	1128.939	30.512	6.102
2346.148	63.409	12.682	1436.305	38.819	7.764	1971.828	53.293	10.659
	Average	9.202		Average	7.407		Average	7.960

Table 9 *Statistical analysis of R(-)-amphetamine*

Parameter	Value			
Table Analyzed				
R(-)-Amphetamine				
One-way analysis of variance				
P value				
P value summary	0.0003			
Are means signif. different? (P < 0.05)	***			
Number of groups	Yes			
F	6			
R squared	6.019			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	1.890			
P value	0.8642			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	df	MS	
Treatment (between columns)	186.4	5	37.29	
Residual (within columns)	260.2	42	6.195	
Total	446.6	47		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	-1.077	0.8652	P > 0.05	-4.332 to 2.179
Control vs 5 mg/ml	-1.399	1.124	P > 0.05	-4.655 to 1.856
Control vs 15 mg/ml	-1.130	0.9076	P > 0.05	-4.385 to 2.126
Control vs 30 mg/ml	2.009	1.614	P > 0.05	-1.246 to 5.265
Control vs 60 mg/ml	3.977	3.196	P < 0.05	0.7218 to 7.233

Table 10 *Statistical analysis of S(+)-amphetamine*

Parameter	Value
Table Analyzed	
Furanyl Fentanyl	
One-way analysis of variance	
P value	0.3883
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	7
F	1.078
R squared	0.1167
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	3.677
P value	0.7203
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS	
Treatment (between columns)	27.01	6	4.502	
Residual (within columns)	204.5	49	4.174	
Total	231.5	55		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	-0.6801	0.6658	P > 0.05	-3.399 to 2.039
Control vs 5 mg/ml	-0.6601	0.6462	P > 0.05	-3.379 to 2.059
Control vs 15 mg/ml	0.8506	0.8327	P > 0.05	-1.869 to 3.570
Control vs 30 mg/ml	-0.8651	0.8469	P > 0.05	-3.584 to 1.854
Control vs 60 mg/ml	0.9295	0.9099	P > 0.05	-1.790 to 3.649
Control vs 120 mg/ml	0.3772	0.3693	P > 0.05	-2.342 to 3.097

3. Zolpidem

Table 11 *Control group testing for Zolpidem*

Control (pixels)	Distance (cm)	Velocity (cm/min)
854.764	23.102	4.620
1164.031	31.460	6.292
1172.595	31.692	6.338
1444.908	39.052	7.810
1048.923	28.349	5.670
841.445	22.742	4.548
1614.997	43.649	8.730
1136.298	30.711	6.142
	Average	6.269

Table 12 *Locomotion Results of Zolpidem*

2 µg/ml	Dis. (cm)	V(cm/min)	*5* µg/ml	Dis. (cm)	V(cm/min)	*15* µg/ml	Dis. (cm)	V(cm/min)
1535.256	41.493	8.299	1369.106	37.003	7.401	1354.395	36.605	7.321
1127.750	30.480	6.096	767.202	20.735	4.147	1137.781	30.751	6.150
904.522	24.447	4.889	1227.200	33.168	6.634	733.287	19.819	3.964
1187.133	32.085	6.417	881.502	23.824	4.765	981.948	26.539	5.308
1269.767	34.318	6.864	717.229	19.385	3.877	611.637	16.531	3.306
736.218	19.898	3.980	1108.863	29.969	5.994	1047.470	28.310	5.662
212.951	5.755	1.151	893.982	24.162	4.832	1367.346	36.955	7.391
814.987	22.027	4.405	1059.402	28.632	5.726	1310.569	35.421	7.084
	Average	5.263		Average	5.422		Average	5.773
30 µg/ml	Dis. (cm)	V(cm/min)	*60* µg/ml	Dis. (cm)	V(cm/min)	*120*µg/ml	Dis. (cm)	V(cm/min)
971.098	26.246	5.249	877.826	23.725	4.745	633.274	17.116	3.423
966.114	26.111	5.222	826.733	22.344	4.469	918.947	24.836	4.967
994.177	26.870	5.374	888.080	24.002	4.800	393.963	10.648	2.130
765.495	20.689	4.138	526.078	14.218	2.844	467.172	12.626	2.525
646.928	17.485	3.497	503.492	13.608	2.722	912.536	24.663	4.933

778.443	21.039	4.208	536.327	14.495	2.899	702.523	18.987	3.797
949.612	25.665	5.133	965.398	26.092	5.218	994.689	26.883	5.377
853.786	23.075	4.615	832.682	22.505	4.501	563.301	15.224	3.045
	Average	4.679		Average	4.025		Average	3.775

Table 13 *Statistical analysis of Zolpidem*

Parameter	Value			
Table Analyzed				
Morphine				
One-way analysis of variance				
P value	0.0067			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	7			
F	3.420			
R squared	0.2952			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	9.762			
P value	0.1350			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	df	MS	
Treatment (between columns)	40.03	6	6.672	
Residual (within columns)	95.59	49	1.951	
Total	135.6	55		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	1.006	1.441	P > 0.05	-0.8529 to 2.865
Control vs 5 mg/ml	0.8467	1.212	P > 0.05	-1.012 to 2.706
Control vs 15 mg/ml	0.4955	0.7095	P > 0.05	-1.364 to 2.355
Control vs 30 mg/ml	1.589	2.276	P > 0.05	-0.2698 to 3.448
Control vs 60 mg/ml	2.244	3.213	P < 0.05	0.3849 to 4.103
Control vs 120 mg/ml	2.494	3.571	P < 0.01	0.6351 to 4.353

4. Barbiturates vs. Methamphetamine

Table 14 *Control group testing for Secobarbital and Methamphetamine*

Control (pixels)	Distance (cm)	Velocity (cm/min)
1509.175	40.789	8.158
1759.200	47.546	9.509
1867.080	50.462	10.092
1641.374	44.361	8.872
1573.614	42.530	8.506
1908.790	51.589	10.318
1794.102	48.489	9.698

1627.194	43.978	8.796
	Average	9.244

Table 15 *Locomotion Results of Secobarbital*

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
1624.069	43.894	8.779	1539.405	41.606	8.321	895.000	24.189	4.838
1595.859	43.131	8.626	726.422	19.633	3.927	1351.473	36.526	7.305
1803.708	48.749	9.750	1757.254	47.493	9.499	1706.325	46.117	9.223
1621.128	43.814	8.763	631.640	17.071	3.414	1679.514	45.392	9.078
1487.213	40.195	8.039	1850.371	50.010	10.002	505.069	13.651	2.730
1691.020	45.703	9.141	1732.711	46.830	9.366	1530.303	41.360	8.272
1269.348	34.307	6.861	1359.089	36.732	7.346	1714.092	46.327	9.265
1720.002	46.487	9.297	0.000	0.000	0.000	0.000	0.000	0.000
	Average	8.657		Average	6.484		Average	6.339
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)	120µg/ml	Dis. (cm)	V(cm/min)
1160.299	31.359	6.272	239.340	6.469	1.294	1391.424	37.606	7.521
1721.872	46.537	9.307	695.329	18.793	3.759	1695.378	45.821	9.164
1375.658	37.180	7.436	1464.029	39.568	7.914	1753.187	47.383	9.477
1570.265	42.440	8.488	605.265	16.359	3.272	1455.044	39.326	7.865
1603.496	43.338	8.668	1752.534	47.366	9.473	952.941	25.755	5.151
103.843	2.807	0.561	1272.239	34.385	6.877	1166.888	31.538	6.308
1378.025	37.244	7.449	1167.506	31.554	6.311	1382.627	37.368	7.474
657.567	17.772	3.554	1068.949	28.891	5.778	1303.897	35.240	7.048
	Average	6.467		Average	5.585		Average	7.501

Table 16 *Locomotion Results of Methamphetamine*

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
1852.990	50.081	10.016	1995.605	53.935	10.787	413.233	11.168	2.234
1642.779	44.399	8.880	1674.196	45.249	9.050	1388.001	37.514	7.503
1649.329	44.576	8.915	1671.929	45.187	9.037	738.305	19.954	3.991
1705.922	46.106	9.221	1308.834	35.374	7.075	1603.410	43.335	8.667
1570.994	42.459	8.492	1512.732	40.885	8.177	1703.966	46.053	9.211
1462.251	39.520	7.904	1593.684	43.073	8.615	1560.139	42.166	8.433
1482.131	40.058	8.012	1550.246	41.899	8.380	1446.163	39.085	7.817
1672.697	45.208	9.042	1749.670	47.288	9.458	1379.000	37.270	7.454
	Average	8.810		Average	8.822		Average	6.914
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)	120µg/ml	Dis. (cm)	V(cm/min)
1850.953	50.026	10.005	1823.583	49.286	9.857	541.932	14.647	2.929
1868.132	50.490	10.098	592.446	16.012	3.202	411.072	11.110	2.222
1748.650	47.261	9.452	1696.385	45.848	9.170	2019.058	54.569	10.914
1520.381	41.091	8.218	1635.865	44.213	8.843	1275.408	34.470	6.894
1624.121	43.895	8.779	1427.232	38.574	7.715	1341.735	36.263	7.253
1877.976	50.756	10.151	1686.167	45.572	9.114	1859.169	50.248	10.050
1712.548	46.285	9.257	1905.412	51.498	10.300	1166.742	31.534	6.307

1986.321	53.684	10.737	431.596	11.665	2.333	1599.277	43.224	8.645
	Average	9.587		Average	7.567		Average	6.902

Table 17 *Statistical analysis of Secobarbital*

Parameter	Value			
Table Analyzed				
R(-)-Amphetamine				
One-way analysis of variance				
P value				
P value summary	0.0231			
Are means signif. different? (P < 0.05)	*			
Number of groups	Yes			
F	7			
R squared	2.735			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	20.28			
P value	0.0025			
P value summary	**			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	75.99	6	12.67	
Residual (within columns)	217.6	47	4.630	
Total	293.6	53		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	0.6789	0.6310	P > 0.05	-2.190 to 3.547
Control vs 5 mg/ml	1.925	1.729	P > 0.05	-1.044 to 4.894
Control vs 15 mg/ml	2.091	1.878	P > 0.05	-0.8777 to 5.061
Control vs 30 mg/ml	2.869	2.667	P < 0.05	0.00057 to 5.737
Control vs 60 mg/ml	3.751	3.486	P < 0.01	0.8827 to 6.620

Table 18 *Statistical analysis of Methamphetamine*

Parameter	Value
Table Analyzed	
Furanyl Fentanyl	
One-way analysis of variance	
P value	0.0305
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	7
F	2.568
R squared	0.2393
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	33.52
P value	P<0.0001
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS	
Treatment (between columns)	61.39	6	10.23	
Residual (within columns)	195.2	49	3.984	
Total	256.6	55		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	0.5256	0.5267	P > 0.05	-2.131 to 3.182
Control vs 5 mg/ml	0.5135	0.5146	P > 0.05	-2.143 to 3.170
Control vs 15 mg/ml	2.422	2.427	P > 0.05	-0.2344 to 5.079
Control vs 30 mg/ml	-0.2513	0.2518	P > 0.05	-2.908 to 2.405
Control vs 60 mg/ml	1.769	1.773	P > 0.05	-0.8874 to 4.426
Control vs 120 mg/ml	2.434	2.439	P > 0.05	-0.2224 to 5.091

5. Cannabinoids

Table 19 *Control group testing for Delta-9-THC and MA-CHMINACA*

Control (pixels)	Distance (cm)	Velocity (cm/min)
2178.315	58.873	11.775
1058.574	28.610	5.722
1673.303	45.224	9.045
1808.978	48.891	9.778
2192.888	59.267	11.853
1812.347	48.982	9.796
1283.724	34.695	6.939
1599.808	43.238	8.648
	Average	9.195

Table 20 *Locomotion Results of Delta-9-THC*

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
1758.087	47.516	9.503	1987.919	53.728	10.746	1805.986	48.810	9.762
1853.495	50.094	10.019	2038.619	55.098	11.020	1680.339	45.415	9.083
1474.257	39.845	7.969	2039.270	55.115	11.023	1772.065	47.894	9.579
1990.676	53.802	10.760	2145.171	57.978	11.596	2056.563	55.583	11.117
1420.439	38.390	7.678	1637.042	44.244	8.849	1399.098	37.813	7.563
1832.716	49.533	9.907	1854.928	50.133	10.027	1521.305	41.116	8.223
1902.743	51.425	10.285	1533.686	41.451	8.290	1994.157	53.896	10.779
1540.406	41.633	8.327	1806.435	48.823	9.765	1581.181	42.735	8.547
	Average	9.306		Average	10.164		Average	9.332
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)	120µg/ml	Dis. (cm)	V(cm/min)
1247.727	33.722	6.744	378.911	10.241	2.048	1257.805	33.995	6.799
1011.226	27.330	5.466	923.499	24.959	4.992	1309.637	35.396	7.079
1069.926	28.917	5.783	1017.648	27.504	5.501	1197.750	32.372	6.474
1375.790	37.184	7.437	1714.472	46.337	9.267	1268.699	34.289	6.858
1759.434	47.552	9.510	774.019	20.919	4.184	1283.036	34.677	6.935

1459.069	39.434	7.887	1560.894	42.186	8.437	1301.395	35.173	7.035
1073.595	29.016	5.803	358.285	9.683	1.937	417.178	11.275	2.255
1388.050	37.515	7.503	1558.747	42.128	8.426	1183.165	31.977	6.395
	Average	7.017		Average	5.599		Average	6.229

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
2023.322	54.684	10.937	2023.322	54.684	10.937	1624.091	43.894	8.779
2074.504	56.068	11.214	2074.504	56.068	11.214	1471.707	39.776	7.955
1757.152	47.491	9.498	1757.152	47.491	9.498	1655.001	44.730	8.946
1564.281	42.278	8.456	1564.281	42.278	8.456	1742.374	47.091	9.418
1797.254	48.574	9.715	1474.440	39.850	7.970	1452.662	39.261	7.852
2028.023	54.811	10.962	2210.664	59.748	11.950	1513.964	40.918	8.184
1727.868	46.699	9.340	1444.783	39.048	7.810	1425.183	38.518	7.704
2140.511	57.852	11.570	1669.260	45.115	9.023	1498.860	40.510	8.102
	Average	10.211		Average	9.607		Average	8.367
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)	120µg/ml	Dis. (cm)	V(cm/min)
1568.743	42.398	8.480	1417.054	38.299	7.660	1145.059	30.948	6.190
1572.282	42.494	8.499	923.882	24.970	4.994	1259.560	34.042	6.808
1646.462	44.499	8.900	1273.434	34.417	6.883	1254.178	33.897	6.779
1470.603	39.746	7.949	1160.646	31.369	6.274	1031.523	27.879	5.576
1286.771	34.778	6.956	1434.114	38.760	7.752	885.854	23.942	4.788
1454.363	39.307	7.861	1343.366	36.307	7.261	1155.534	31.231	6.246
1672.500	45.203	9.041	704.550	19.042	3.808	1040.458	28.120	5.624
1261.909	34.106	6.821	1182.887	31.970	6.394	1253.807	33.887	6.777
	Average	8.063		Average	6.378		Average	6.099

Table 21 *Locomotion Results of MA-CHMINACA*

Table 22 *Control group testing for JWH 133*

Control (pixels)	Distance (cm)	Velocity (cm/min)
1181.640	31.936	6.387
1651.426	44.633	8.927
1323.655	35.774	7.155
1607.369	43.442	8.688
1506.764	40.723	8.145
1641.221	44.357	8.871
1601.333	43.279	8.656
1427.405	38.579	7.716
	Average	8.068

Table 23 *Locomotion Results of JWH 133*

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
1680.696	45.424	9.085	1958.246	52.926	10.585	1663.083	44.948	8.990
1661.127	44.895	8.979	1619.705	43.776	8.755	1542.733	41.695	8.339
1686.363	45.577	9.115	1130.185	30.546	6.109	1282.366	34.659	6.932
866.477	23.418	4.684	1827.658	49.396	9.879	1410.852	38.131	7.626
1481.388	40.038	8.008	1005.200	27.168	5.434	1585.632	42.855	8.571
1587.464	42.904	8.581	1323.309	35.765	7.153	1740.309	47.035	9.407
1572.930	42.512	8.502	1746.236	47.196	9.439	1226.343	33.144	6.629
1060.532	28.663	5.733	1542.477	41.689	8.338	1615.341	43.658	8.732
	Average	7.836		Average	8.211		Average	8.153
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)	120µg/ml	Dis. (cm)	V(cm/min)
1477.312	39.927	7.985	1341.700	36.262	7.252	1480.387	40.010	8.002
1402.658	37.910	7.582	1369.700	37.019	7.404	1329.169	35.923	7.185
1539.467	41.607	8.321	1627.635	43.990	8.798	1731.299	46.792	9.358
1587.227	42.898	8.580	1341.753	36.264	7.253	1585.765	42.859	8.572
954.136	25.787	5.157	1343.107	36.300	7.260	1693.902	45.781	9.156
1651.795	44.643	8.929	824.790	22.292	4.458	1611.875	43.564	8.713
1882.217	50.871	10.174	1343.840	36.320	7.264	1333.124	36.030	7.206
1500.488	40.554	8.111	1664.391	44.984	8.997	1259.830	34.049	6.810
	Average	8.105		Average	7.336		Average	8.125

Table 24 *Statistical analysis of Delta-9-THC*

Parameter	Value
Table Analyzed	
R(-)-Amphetamine	
One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	7
F	8.412
R squared	0.5074
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	10.98
P value	0.0889
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	154.9	6	25.81
Residual (within columns)	150.3	49	3.068
Total	305.2	55	

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	-0.1115	0.1273	P > 0.05	-2.443 to 2.220
Control vs 5 mg/ml	-0.9700	1.108	P > 0.05	-3.301 to 1.361
Control vs 15 mg/ml	-0.1371	0.1566	P > 0.05	-2.469 to 2.194
Control vs 30 mg/ml	2.178	2.487	P > 0.05	-0.1536 to 4.509
Control vs 60 mg/ml	3.596	4.105	P < 0.01	1.264 to 5.927

Table 25 *Statistical analysis of MA-CHMINACA*

Parameter	Value
Table Analyzed	
Furanyl Fentanyl	
One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	7
F	11.75
R squared	0.5900
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	15.70
P value	0.0155
P value summary	*
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	118.1	6	19.68
Residual (within columns)	82.06	49	1.675
Total	200.1	55	

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	-1.017	1.572	P > 0.05	-2.739 to 0.7055
Control vs 5 mg/ml	-0.4128	0.6379	P > 0.05	-2.135 to 1.310
Control vs 15 mg/ml	0.8270	1.278	P > 0.05	-0.8955 to 2.549
Control vs 30 mg/ml	1.131	1.748	P > 0.05	-0.5913 to 2.854
Control vs 60 mg/ml	2.816	4.352	P < 0.01	1.094 to 4.539
Control vs 120 mg/ml	3.096	4.785	P < 0.01	1.374 to 4.818

Table 26 *Statistical analysis of JWH133*

Parameter	Value
Table Analyzed	
Furanyl Fentanyl	
One-way analysis of variance	
P value	0.8700
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	7
F	0.4083
R squared	0.04762
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	6.085
P value	0.4137
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS	
Treatment (between columns)	4.510	6	0.7517	
Residual (within columns)	90.21	49	1.841	
Total	94.72	55		
Dunnett's Multiple Comparison Test	**Mean Diff.**	**q**	**P value**	**95% CI of diff**
Control vs 2 mg/ml	0.2322	0.3423	P > 0.05	-1.574 to 2.038
Control vs 5 mg/ml	-0.1434	0.2113	P > 0.05	-1.949 to 1.663
Control vs 15 mg/ml	-0.08512	0.1255	P > 0.05	-1.891 to 1.721
Control vs 30 mg/ml	-0.03675	0.05417	P > 0.05	-1.843 to 1.769
Control vs 60 mg/ml	0.7324	1.080	P > 0.05	-1.074 to 2.538
Control vs 120 mg/ml	-0.05713	0.08420	P > 0.05	-1.863 to 1.749

6. Hallucinogen

Table 27 *Control group testing for 25I-NBOMe*

Control (pixels)	Distance (cm)	Velocity (cm/min)
1167.658	31.558	6.312
1030.483	27.851	5.570
1005.345	27.171	5.434
1083.653	29.288	5.858
1183.934	31.998	6.400
986.432	26.660	5.332
1159.662	31.342	6.268
1138.884	30.781	6.156
	Average	5.916

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
1070.667	28.937	5.787	945.242	25.547	5.109	1249.275	33.764	6.753
1205.877	32.591	6.518	952.838	25.752	5.150	1380.443	37.309	7.462
1407.140	38.031	7.606	992.565	26.826	5.365	1271.871	34.375	6.875
1390.050	37.569	7.514	1178.479	31.851	6.370	1267.694	34.262	6.852
1133.514	30.636	6.127	1077.333	29.117	5.823	1392.521	37.636	7.527
846.560	22.880	4.576	1238.313	33.468	6.694	952.688	25.748	5.150
1304.741	35.263	7.053	1045.929	28.268	5.654	1148.470	31.040	6.208
759.305	20.522	4.104	1005.453	27.174	5.435	1309.019	35.379	7.076
	Average	6.161		Average	5.700		Average	6.738
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)	120 µg/ml	Dis. (cm)	V(cm/min)
1053.819	28.482	5.696	1124.263	30.385	6.077	1353.604	36.584	7.317
1236.829	33.428	6.686	565.606	15.287	3.057	509.764	13.777	2.755
1095.240	29.601	5.920	1079.216	29.168	5.834	896.114	24.219	4.844
1187.403	32.092	6.418	1035.955	27.999	5.600	1094.719	29.587	5.917
1228.623	33.206	6.641	224.044	6.055	1.211	1323.488	35.770	7.154
1015.078	27.435	5.487	1359.039	36.731	7.346	928.486	25.094	5.019

1204.598	32.557	6.511	905.969	24.486	4.897	1194.216	32.276	6.455
988.438	26.715	5.343	576.873	15.591	3.118	901.167	24.356	4.871
	Average	6.088		Average	4.643		Average	5.542

Table 28 *Locomotion Results of 25I-NBOMe*

Table 29 *Statistical analysis of 25I-NBOMe*

Parameter	Value			
Table Analyzed				
Furanyl Fentanyl				
One-way analysis of variance				
P value	0.0338			
P value summary	*			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	7			
F	2.511			
R squared	0.2351			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	25.89			
P value	0.0002			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	20.14	6	3.357	
Residual (within columns)	65.51	49	1.337	
Total	85.65	55		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	-0.2444	0.4227	P > 0.05	-1.783 to 1.295
Control vs 5 mg/ml	0.2163	0.3740	P > 0.05	-1.323 to 1.755
Control vs 15 mg/ml	-0.8216	1.421	P > 0.05	-2.361 to 0.7174
Control vs 30 mg/ml	-0.1715	0.2966	P > 0.05	-1.710 to 1.367
Control vs 60 mg/ml	1.274	2.203	P > 0.05	-0.2652 to 2.813
Control vs 120 mg/ml	0.3748	0.6482	P > 0.05	-1.164 to 1.914

7. Cocaine

Table 30 *Control group testing for Cocaine*

Control (pixels)	Distance (cm)	Velocity (cm/min)
1486.437	40.174	8.035
1358.761	36.723	7.345
1453.782	39.291	7.858

1328.484	35.905	7.181
938.139	25.355	5.071
703.453	19.012	3.802
1271.042	34.352	6.870
1378.464	37.256	7.451
	Average	6.702

Table 20 *Locomotion Results of Cocaine*

2 µg/ml	Dis. (cm)	V(cm/min)	5 µg/ml	Dis. (cm)	V(cm/min)	15 µg/ml	Dis. (cm)	V(cm/min)
1359.977	36.756	7.351	960.626	25.963	5.193	1477.018	39.919	7.984
1620.481	43.797	8.759	1495.746	40.426	8.085	903.239	24.412	4.882
1198.277	32.386	6.477	1499.073	40.515	8.103	1408.461	38.067	7.613
1411.882	38.159	7.632	1672.125	45.193	9.039	1706.734	46.128	9.226
1011.704	27.343	5.469	1575.591	42.584	8.517	1611.692	43.559	8.712
1237.910	33.457	6.691	1648.193	44.546	8.909	1105.934	29.890	5.978
1558.325	42.117	8.423	1379.357	37.280	7.456	1218.695	32.938	6.588
1656.106	44.760	8.952	1337.875	36.159	7.232	1934.794	52.292	10.458
	Average	7.469		Average	7.817		Average	7.680
30 µg/ml	Dis. (cm)	V(cm/min)	60 µg/ml	Dis. (cm)	V(cm/min)	120 µg/ml	Dis. (cm)	V(cm/min)
1943.605	52.530	10.506	821.766	22.210	4.442	789.005	21.324	4.265
694.760	18.777	3.755	717.129	19.382	3.876	913.217	24.682	4.936
1374.793	37.157	7.431	1661.791	44.913	8.983	918.272	24.818	4.964
1252.363	33.848	6.770	1047.190	28.302	5.660	886.322	23.955	4.791
963.174	26.032	5.206	933.964	25.242	5.048	1376.032	37.190	7.438
794.892	21.484	4.297	1385.082	37.435	7.487	1036.867	28.023	5.605
1610.140	43.517	8.703	674.261	18.223	3.645	1116.585	30.178	6.036
465.684	12.586	2.517	784.381	21.199	4.240	1337.762	36.156	7.231
	Average	6.148		Average	5.423		Average	5.658

Table 29 *Statistical analysis of 25I-NBOMe*

Parameter	Value
Table Analyzed	
Furanyl Fentanyl	
One-way analysis of variance	
P value	0.0277
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	7
F	2.623
R squared	0.2431
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	8.152

P value	0.2272			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	df	MS	
Treatment (between columns)	46.57	6	7.761	
Residual (within columns)	145.0	49	2.959	
Total	191.6	55		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Control vs 2 mg/ml	-0.7676	0.8925	P > 0.05	-3.057 to 1.522
Control vs 5 mg/ml	-1.115	1.296	P > 0.05	-3.405 to 1.175
Control vs 15 mg/ml	-0.9785	1.138	P > 0.05	-3.268 to 1.311
Control vs 30 mg/ml	0.5535	0.6435	P > 0.05	-1.736 to 2.843
Control vs 60 mg/ml	1.279	1.487	P > 0.05	-1.011 to 3.569
Control vs 120 mg/ml	1.043	1.213	P > 0.05	-1.246 to 3.333