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Identification of the New Synthetic Opioid Bucinnazine and its Metabolites using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

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ABSTRACT

In the past 20 years, the rate of opioid use increased drastically in the United States. The country has experienced an enormous number of deaths caused by opioid overdose, opioid use disorder (OUD), as well as other harms as a consequence of the high numbers of opioid prescriptions. Fentanyl, a well-known synthetic opioid, has been a contributor to the opioid crisis since 2013 due to its popularity in clinical and illicit use. It has a high potency, which makes it an effective analgesic but a dangerous illicit substance. A new synthetic opioid, bucinnazine, has recently become a new black-market opioid that is not scheduled in the U.S. Bucinnazine is structurally distant from fentanyl and its main analogs, but it demonstrates pharmacological properties that are similar to fentanyl. New synthetic opioids, including bucinnazine, are difficult to identify in routine testing due to the constant changing structures. Despite the clinical history of bucinnazine, little data is available concerning its metabolism. A method for the determination of bucinnazine was devised using liquid chromatography tandem mass spectrometry (LC-MS/MS). Precursor and product ions were identified and used to develop a rapid and accurate method for identification of bucinnazine and its metabolites in different samples.

Key words: bucinnazine, LC-MS/MS, metabolism

INTRODUCTION

The Opioid Epidemic

In the past 20 years, the rate of opioid use increased drastically in the United States, and the country has experienced an enormous increase in deaths from opioid overdose, opioid use disorder (OUD), and other harms as a consequence of the high numbers of opioid prescriptions. In 2016, a total of 63,632 drug overdose deaths occurred, corresponding to a 21.4% increase compared to 2015[1-3]. About two thirds (66.4%) of drug overdose deaths in 2016 involved prescription opioids, illicit opioids, or a combination of both, resulting in an increase of 27.7% compared to data from 2015[1,2]. In addition, the rate of drug overdose deaths involving synthetic opioids nearly doubled in a single year[2]. In 2018, approximately 70% of the 67,367 overdose deaths were due to the use of opioids, and from 2017 to 2018, the synthetic opioid-involved deaths had increased by 10%, even as other opioid-involved deaths decreased slightly[3]. In 2019, over 64,000 drug overdose deaths were reported, and in 2020 there was a 29.9% increase[4]. Approximately 74% of the drug overdose deaths that occurred in 2020 were due to opioids[4].

A rapid response to these fast outbreaks of synthetic opioids can be very difficult. Oftentimes any data about the introduction of new substances is only collected after an overdose death has occurred and been brought to the attention of healthcare professionals[5]. There are several early warning systems in place that are set up so that any seizures of illicit synthetic opioids are processed and identified[6]. In 2013, the United Nations Office for Drugs and Crime (UNODC) launched the UNODC Early Warning Advisory (EWA) to monitor, analyze, and report trends on new psychoactive substances (NPS), including synthetic opioids, at the global level, as a basis for

effective evidence-based policy responses[7]. Usually, the notifications and reports on these new substances that are compiled take time, and in that time the epidemic increases[8].

Fast response and efficient monitoring are vital tools for the identification of as many different analogs of the different synthetic opioids as possible. In order to decrease the time it takes to identify new drugs and determine the danger that they may present, the development of fast and reliable identification methods for these substances is mandatory.

Synthetic Opioids

Synthetic opioids are a subclass of Novel Psychoactive Substances (NPS) that are defined by the United Nations Office on Drugs and Crime (UNODC) as "substances of abuse, either in pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs of the 1971 Convention on Psychotropic Substances, but which may pose a public health threat"[9]. These are often marketed as "legal highs" or research chemicals and are often difficult to track and/or study. There are several subclasses of NPS, including synthetic cannabinoids, synthetic cathinones, phenethylamines, plant based substances such as Kratom, tryptamines, aminoindanes, phencyclidine-type substances, and synthetic opioids[9].

Synthetic opioids are opioids that share pharmacological characteristics with morphine, but are synthesized in laboratory, and do not share a core structure with morphine. The initial syntheses of many synthetic opioids are the result of legitimate research attempting to determine safer and more effective alternatives to morphine as analgesics for pain relief[10,11]. The synthesis methods for these synthetic opioids are then available online, but the synthetic opioids themselves are not approved for clinical use. Illicit drug manufacturers are able to take advantage of these available protocols and use them to create analogs that are highly physiologically active[12]. These analogs are often not scheduled[13].

The most popular and well known of the synthetic opioids is fentanyl. Fentanyl, shown in Figure 1, is an opioid first synthesized by Paul Janssen, of Janssen Pharmaceutica, in 1959, in an attempt to develop a better and safer analgesic medication[14,15]. It is a rapid acting, Schedule II drug that is typically used in anesthesia and analgesia, and is 100 times more potent than morphine[14,12,16]. Fentanyl and its analogs are classified as phenylpiperidines. Fentanyl and other synthetic opioids work by binding to receptors in the brain, specifically the μ-opioid receptor. The μ-opioid receptor is a subtype receptor that is responsible for the variety of effects presented after opioid use, including the desired ones of analgesia and euphoria as well as side effects such as muscular rigidity, respiratory depression, or apnea[17]. The other opioid receptors subtypes are the δ-opioid receptor and the κ-opioid receptor. The δ-opioid receptor is a subtype that has strong analgesic effects and antidepressant-like effects[18,19]. The κ-opioid receptor is a subtype that is potentially hallucinogenic, although it may reduce stress responses, reduce drug craving, and remediate depressive states, as well as playing a role in reward and mood processes[19,20]. As opioids bind to the receptors, the G-proteins coupled to the receptors are activated, which in turn inhibits cAMP.

Piperazines

One popular compound in the piperazines class is MT-45, shown in Figure 2. This compound was initially synthesized in the 1970s in Japan by the Dainippon Pharmaceutical Company[21]. Like other synthetic opioids, MT-45 was intended to be an alternative to morphine, as it has a similar potency to morphine. However, it did not move to clinical trials in humans, and so there are no published studies that discuss safety for human use of MT-45[22]. It was first reported on the black market in 2013 in cases also involving synthetic cannabinoids and synthetic cathinones. It has since been linked to overdose including reports of deaths in the United States

and Europe. MT-45 was scheduled in the United States in 2017 as a Schedule 1 substance due to its potential for physical dependence and lack of clinical use in the U.S[22].

Cinnamylpiperazines

Cinnamylpiperazines are a subclass of piperazines that have gained attention in recent years as several substances in this class were reported by the UNODC's early warning advisory[23]. In 2019, three structural analogs in the class of drug were reported in Canada and Sweden, followed by the United States in 2020[23]. The parent compound of these analogs is called bucinnazine, or AP-237. Bucinnazine is structurally distant from fentanyl and its main analogs, but it also has several similarities. It is considered a Novel Psychoactive Substance, and it is currently unscheduled in the United States[24]. Bucinnazine was initially characterized around 1970, testing of its properties, effects, and usefulness is still ongoing[24,25]. In the world of healthcare, bucinnazine has replaced fentanyl and morphine as a narcotic analgesic in advanced cancer patients and those undergoing trauma therapy in China[26]. Bucinnazine has been reported, as with other synthetic opioids, that repeated use can cause the central nervous system to become paralyzed, which can cause reliance on the drug[27].

Chemistry of Bucinnazine

Bucinnazine (1-butyryl-4-cinnamylpiperazine)[24], shown in Figure 3, is a synthetic opioid with a molecular weight of 272.4 g/mol. It is a white crystalline powder that is easily soluble in water and has been shown to possess good analgesic activity[25]. Bucinnazine has been categorized as a Novel Psychoactive Substance due to its potential for abuse.

The analgesic effect of bucinnazine is approximately one third that of morphine, and has a lower addiction rate[27]. Opioids tend to be basic, with a pKa between 7.5 and 10.9[1]. Bucinnazine, like other opioids, has high lipophilicity, which allows for rapid diffusion through membranes.

This includes the blood-brain barrier (BBB), which is one reason why these synthetic opioids are so potent. The defined daily dose of bucinnazine was determined to be 30 mg as a bucinnazine hydrochloride tablet, or 0.1 g as a bucinnazine hydrochloride injection[27].

Bucinnazine has three currently known analogs, 2-methyl AP-237, *para*-methyl AP-237, and AP-238, shown in Figure 4. The difference in structure between bucinnazine and these three analogs is the addition of a methyl group on different parts of either the piperazine ring or the benzene ring. 2-methyl AP-237 was found on the black market just before bucinnazine in 2019, and has since been identified in several cases involving unknown powders and overdose deaths[28].

Pharmacology of Bucinnazine

Bucinnazine is part of the piperazine class of new synthetic opioids. It is one of the most potent compounds in this series, alongside other piperazines such as MT-45, AD-1211, and the methylated derivative of bucinnazine, 2-methyl-AP-237[29]. The therapeutic index of bucinnazine is high compared to other analgesics, which makes it a good alternative for pain treatment. It is the analgesic of choice in China for this reason, as it is an effective alternative to morphine. Similarly to other synthetic opioids, bucinnazine is a μ-selective opioid, which means that it primarily binds to the μ-opioid receptor in the brain[10]. However, most other piperazines act on the dopamine, serotonin, and norepinephrine neurotransmitters, and so it is likely that bucinnazine also activates the protein G and β-arrestin pathways of the brain[30].

Bucinnazine contains a piperazine ring. This structural aspect has specific pharmacological properties that are somewhat similar to other opioids. Bucinnazine acts as on the central nervous system as a depressant and a stimulant. There is possible ganglionic blocking and anti-serotonin activity, and bucinnazine has some tranquilizing effects in addition to showing analgesic activity. Bucinnazine caused behaviors similar to those of morphine in a study in rats, dogs, and cats[25].

The maximum sublethal dose (MSLD) in rats was determined to be 100mg/kg intraperitoneal and intramuscular, and 316 mg/kg oral and subcutaneous.

Metabolism of Bucinnazine

Not much is known about the metabolism of new synthetic opioids, including that of bucinnazine. Baba, Morishita, and Terayama (1973, 1975, 1978) described a potential metabolic pathway for bucinnazine consisting of *p*-hydroxylation and N-dealkylation as the main reactions[31,32,33,34]. In these studies, seven different metabolites were found: 1-butyryl-4-(4-hydroxycinnamyl)piperazine, 1-cinnamylpiperazine, 1-(4-hydroxycinnamyl)piperazine, benzoic acid, 4-hydroxybenzoic acid, hippuric acid, and 4-hydroxyhippuric acid. Most of the methods used in these studies, while effective, are now out-of-date, and so further research into the metabolism of bucinnazine is important in order to obtain a better understanding of the substance and the potential effects it may have. The metabolism of a drug is also important in the identification of the biomarkers of exposure in biological matrices.

In order to determine the metabolic pathway of bucinnazine, *in vitro* studies can be performed using rat or human liver microsomes. Microsomes are structures derived from pieces of endoplasmic reticulum (ER) formed during tissue homogenization. They are prepared by differential centrifugation and contain cytochrome P450 enzymes (CYPs). They are used as an enzyme source for determining metabolic profiles because they express drug-metabolizing enzymes[35]. The metabolic pathway of *p*-hydroxylation occurs when a hydroxyl group is added to bucinnazine through the chemical processes that take place in the liver (or under *in vitro* conditions). The metabolic pathway of *N*-dealkylation occurs when an alkyl group is removed from bucinnazine through the chemical processes that take place in the liver (or under *in vitro*

conditions). Enzymes convert bucinnazine into metabolites that are more water soluble, so that in the body they can be more easily eliminated.

Analytical Methods

Several analytical methods exist in the literature to identify bucinnazine as powders and in biological matrices. Many of the original methods of analysis include a carbon isotope labeling procedure, followed by high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometer-multiple ion detector (GC-MS-MID)[31,32,33,36,]. One study from 2018 utilizes surface-enhanced Raman spectroscopy (SERS) to detect bucinnazine in water and urine samples[37]. A third method of analysis uses high-performance liquid chromatography-diode array detector (HPLC-DAD) to identify bucinnazine and other unknown compounds[38,39]. Each of these methods are useful for the identification but are not efficient or as sensitive as other methods of analysis that are currently available. In particular, liquid chromatography tandem mass spectrometry (LC-MS/MS) has become more popular in recent years as a useful method for the detection and identification of narcotic opioids and other misused substances[14,16,40,41].

Hypothesis and Aims of the Project

The hypothesis of this project poses the question: can bucinnazine be identified in unknown samples using metabolites and biomarkers? Bucinnazine has been shown to be present on the black market, and so the development of method for screening and identification is vital. There are three main aims of this project to answer the hypothesis. First it is to develop an LC-MS/MS method to screen for bucinnazine and other fentanyl analogs. Second is to develop a method to test the *in vitro* metabolism of bucinnazine and to identify potential metabolites. The third and final aim is to utilize the developed LC-MS/MS method to quantify bucinnazine samples and screen for bucinnazine metabolites.

MATERIALS AND METHODS

Reagents

Methanol, acetonitrile, dimethylsulfoxide (DMSO), ammonium formate, 0.1% formic acid, phosphate buffer pH 7.4, Gibco Sprague-Dawley rat liver microsomes (20 μg protein/mL), reduced nicotinamide adenine dinucleotide phosphate (NADPH), sodium hydroxide, hydrochloric acid, ethyl acetate, and sodium sulfate were purchased from Fisher Scientific (Waltham, MA). A primary reference standard of bucinnazine was purchased from Cayman Chemical (Ann Arbor, MI), along with the internal standard of fentanyl-D_5 from Cerilliant (Round Rock, TX) and was utilized for quality assurance and quality control purposes. Unknown powder samples suspected to contain bucinnazine were obtained from the Virginia Commonwealth University Health System Hospital. The powder samples were submitted by a patient in the Substance Abuse Therapy program at VCU Health. The patient had purchased the samples from two different online vendors and reported two different experiences after ingesting the powders.

Standard Preparation

Bucinnazine standards were obtained as a powder and were dissolved in methanol to a concentration of 1 mg/mL to make a stock solution of bucinnazine. This stock solution was then diluted to the concentrations of 1 ng/mL, 2.5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 75 ng/mL and 100 ng/mL in the mobile phase starting conditions, which were 65% of 5 mM ammonium formate in water, 0.1% formic acid, and 35% methanol. Control standards for quality control/quality assurance purposes were also prepared, at concentrations of 3 ng/mL, 30 ng/mL, and 80 ng/mL, and were run alongside the calibration standards. Fentanyl-d_5 was used as an internal standard at a concentration of 25 ng/mL. An internal standard blank with fentanyl-d_5 and

the mobile phase starting conditions was prepared, as well as a double blank. These standards were run on the LC-MS/MS and GC-MS.

Unknown Powder Sample Preparation

Unknown powder samples obtained from VCU Health System Hospital were used to demonstrate the application of the LC-MS/MS method. The powder samples were first weighed and the mass was recorded. A small portion of the sample was separated out, weighed, and then dissolved in methanol to a concentration of approximately 1 mg/mL for sample 1 and 0.5 mg/mL for sample 2, depending on the amount of available powder. Some powder was left over for additional analysis as needed. These samples were then diluted to a concentration of 20 µg/mL and run on the DART-MS, GC-MS, and LC-MS/MS. Visual examination of the two powder samples showed several differences in their physical properties. A small sample of both powders was individually taken and then placed on a glass microscope slide to view under a Leica DM750P (Leica Microsystems, Buffalo Grove, IL) compound microscope. Each compound was viewed under 100x, 200x and 400x using light field microscopy. The first unknown powder, 20210226-001 "1", was not able to be viewed under 400x, as the crystals were too large for the working distance of the microscope. The second unknown powder, 20210226-001 "2", was able to be viewed under 100x, 200x, and 400x.

Metabolism Study

Metabolites of bucinnazine were formed *in vitro* and then used as proof of applicability on the developed LC-MS/MS method[35]. Metabolites were formed by first preparing a solution of 10 µM of bucinnazine in DMSO. All preparation was done on ice until the metabolites were ready to be cooked to prevent premature activation or degradation of the reagents. In a tube on ice, 50 µL of 1 M phosphate buffer solution, pH 7.4 was combined with 12.5 µL of Sprague-Dawley rat

microsomes (20 mg protein/mL) and 5 µL of the 10 µM bucinnazine solution. To this tube was then added 417.5 µL of DI water. Pre-incubation of the tube was then performed, placing it in a water bath at 37℃ for 5 minutes to equalize the temperatures of all the reagents. Once pre-incubation was complete, 15 µL of 40 µM NADPH was added to start the reaction. This was repeated for four different tubes. The tubes were vortexed to mix, and then were placed in a shaking water bath at 37℃ for 0 minutes, 30 minutes, 90 minutes, and 180 minutes, to observe the time course of the formation of bucinnazine metabolites. The reaction was terminated by adding 1 mL of ice-cold acetonitrile with 1 µg/mL of the fentanyl-d_5 internal standard to the tube. A set of four control samples was run alongside the four samples, with all reagents except for bucinnazine. The tubes were then vortexed and centrifuged, and then 100 µL of the supernatant was transferred directly to LC-MS/MS vials for analysis while 200 µL of the supernatant was transferred to clean tubes for a basic and an acidic extraction for analysis on the GC-MS[42]. The basic extraction of the sample was performed by using 0.1 N sodium hydroxide to basify the 200 µL sample aliquot, and then by adding 600 µL of ethyl acetate to the tube. This was vortexed, centrifuged, and then the supernatant was transferred to a new tube. Sodium sulfate was added to the supernatant to ensure all water was extracted, and then the sample was dried down under nitrogen and then reconstituted in methanol. For the acidic extraction, 0.1 N hydrochloric acid was added to a separate 200 µL aliquot of the sample, followed by 600 µL of ethyl acetate. This was vortexed, centrifuged, and then the supernatant was transferred to a new tube. Sodium sulfate was added to the supernatant to ensure all water was extracted, and then the sample was dried down under nitrogen and then reconstituted in methanol. Extracted samples were then transferred to GC-MS vials for analysis.

LC-MS/MS Parameters

A Shimadzu LCMS 8050 (Shimadzu Corporation, Kyoto, Japan) LC-MS/MS was used for analysis of the samples. Two mobile phases were used in a gradient, where mobile phase A consisted of 5 mM ammonium formate and 0.1% formic acid in deionized water, and mobile phase B was 100% methanol. The total run time of the method was 8 minutes, and the gradient was as follows: started with 35% B, then increased to 50% B over 2.50 minutes, increased to 90% B over 3 minutes, and then immediately returned to 35% B holding for the remaining 2.5 minutes. The column used was an Agilent InfinityLab Poroshell HPH C18 column, 4.6 mm x 100 mm, 2.7 micron (Agilent, Santa Clara, CA). The initial flow rate was 0.5 mL/min. The injection volume used was 2 μL, and a dwell time of 1 ms was used to enhance peak shape. The transition ions, collision energy, and Q1 and Q3 pre-bias are listed in Table 1. The LC-MS/MS was run in positive mode.

Method Validation Parameters

Validation of the LC-MS/MS method was performed according to the ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology. The validation parameters used were those for quantitative analysis, listed in section 7.5 of Standard 036. This includes calculations of bias and precision, building a calibration model, performing carryover studies, interference studies, and calculation of the limit of detection and the limit of quantitation. The acceptable percent bias of the standards and associated QCs of the standards run as a calibration curve for method validation is within ±20%. Within-run precision for each of the three runs performed must have a %CV within ±20%. Within-run precision assesses the preparation of the QCs run in triplicate for each run. Between-run precision for each of the three runs performed must have a %CV within ±20%. Between-run precision assesses the preparation of samples over different days. No other parameters were necessary to test in this case, as no biological matrices

were used in this research. Method validation was only performed on the developed LC-MS/MS method, as this was the only method used for quantitation.

DART-MS Parameters

Samples were screened on a Jeol JMS-T100LC ionSense DART with an AccuTOF MS (Jeol, Tokyo, Japan). Powder samples were dissolved in methanol according to the sample preparation procedure, and then held in front of the sample injection port in the DART-MS instrument. The temperature was 300℃, the ion source setting was 1 mm, and the DART-MS was operated in positive mode. Calibration of the DART-MS was performed using a PEG 600 solution, and QA/QC of the instrument was insured by using a LOCK solution consisting of cocaine, methamphetamine, and nefazodone with low, mid, and high mass-to-charge ratios.

GC-MS Parameters

Samples were additionally screened on a Shimadzu GC-MS QP-2020 instrument (Shimadzu Corporation, Kyoto, Japan). The column used was an Agilent HP-5MS 30 m x 0.250 mm x 0.250 μm column (Agilent, Santa Clara, CA). The injection temperature was 250℃, the column oven temperature was 70℃ for 1 minute and then was ramped to 300℃ over 15 minutes, and the total run time of the method was 26.33 minutes. The ion source temperature was 250℃. The GC was in splitless mode, and the MS was in SCAN mode with a range of 40.00 *m/z* to 550.00 *m/z*. Powdered samples in methanol at a concentration of 20 μg/mL and bucinnazine metabolites obtained *in vitro* were injected on the instrument.

RESULTS

Method Validation

The method validation was evaluated according to the ASB Standards for Method Validation in Forensic Toxicology. The parameters that were tested are listed in Table 2. The limit of detection and the limit of quantification were determined to be 1.01 ng/mL and 3.40 ng/mL, respectively. No interference was observed from the matrix, and no carryover from the highest concentration standard was observed in any of the six blanks run consecutively after the highest concentration standard. A calibration curve (Figure 5) with seven standards and nine QCs was run over a period of three days, and then the averages of the standards for all the runs were used to develop a linear equation to validate the method.

The residuals analyzed for the calibration model of bucinnazine demonstrate that a linear regression model is acceptable for the calibration model, as the standardized residuals are within ± 3 standard deviations of the observed peak areas obtained (Table 3, Figure 6). All the standards run over the three days fall within the acceptable range for percent bias (Table 4), and all the QCs run with the standards over three days also fall within the acceptable range for percent bias. Both the within-run precision (Table 5) and the between-run precision (Table 6) of the QC samples falls within the acceptable range of $\pm 20\%$ for the %CV.

Metabolism Study

The LC-MS/MS run of the bucinnazine metabolites demonstrates practical use of the developed method. A screening of the metabolites on the LC-MS/MS was performed, and quantitation of bucinnazine in the samples demonstrated the decrease in the amount of bucinnazine over time, supporting the formation of metabolites. A peak on the LC-MS/MS chromatograms at

the expected retention time and with the expected ions was identified as bucinnazine. Two metabolites were observed on the LC-MS/MS, identified potentially as 1-cinnamylpiperazine and 1-butyryl-4-(4-hydroxycinnamyl)piperazine, based on the mass spectra obtained from the peaks identified as a metabolite (Figure 7, Figure 8). A time course study of the formation of 1-cinnamylpiperazine and 1-butyryl-4-(4-hydroxycinnamyl)piperazine was performed. Within the time course, the qualitative formation of 1-cinnamylpiperazine and 1-butyryl-4-(4-hydroxycinnamyl)piperazine could be observed on the LC-MS/MS as the peak area of bucinnazine decreased over time while the peak areas identified as 1-cinnamylpiperazine and 1-butyryl-4-(4-hydroxycinnamyl)piperazine increased over time (Figure 9).

To confirm the identity of the metabolites, a standard of 1-cinnamylpiperazine and 1-butyryl-4-(4-hydroxycinnamyl)piperazine would need to be obtained, but could not be obtained within the scope of this project.

In the absence of standards, the spectral matching procedure is normally applied for the identification of metabolites by LC-MSMS. This method mimics the manual verification of metabolites identity using the MS/MS spectrum. Instead of acquiring the MS/MS spectrum of the authentic compound each time, previously acquired MS/MS spectra of authentic compounds are assembled in a spectral library and used to compare with the spectra acquired from biological samples. The disadvantage of this method relies on the fact that for MS/MS spectral matching, the acquired spectra depend on the equipment acquisition settings that can vary from one instrument to another[43].

The metabolites obtained *in vitro* were screened using GC-MS. Both the acid extracted, and base extracted samples were analyzed on the GC-MS aiming for the identification of metabolites previous described in the literature. No metabolites were observed with the acid

extracted samples. The compound 1-cinnamylpiperazine was observed in the base extracted samples and identified as a potential metabolite based on the mass spectrum obtained from the peak (Figure 10, Figure 11). A time course study of the formation of the single bucinnazine metabolite was performed. Within the time course, the qualitative formation of the 1-cinnamylpiperazine metabolite could be observed on the GC-MS as the peak area of bucinnazine decreased over time while the peak areas identified as 1-cinnamylpierazine increased over time. The formation course of 1-cinnamylpiperazine is shown in Figure 12. The decrease in the peak area of bucinnazine in the sample is depicted by the blue line, and the increase in the peak area of the suspected metabolite is depicted by the orange line.

Unknown Powder Samples

Visual examination of the two powder samples showed several differences in their physical properties. Both were white in color, but 20210226-001 "1" was much more crystalline in nature and had a greater amount of powder present in the bag, while 20210226-001 "2" was very fine, with little substance left in the bag, both shown in Figure 13. Under the microscope, 20210226-001 "1" was clearly a large, crystalline substance, shown in Figure 14, though no other significant microscopic properties were observed. The second sample, 20210226-001 "2" was a small, slightly crystalline substance under the microscope, shown in Figure 15, and no other significant microscopic properties were observed.

The screening of the unknown powders on the DART-MS resulted in a list of compounds for the presumptive identification of the unknown powders. For sample 20210226-001 "1", the highest peak showed a mass of 222.1 *m/z* (Figure 16). The library search (SWGDRUG Library) showed the structural isomer of fluoro-deschloroketamine, possibly 2-, 3-, or 4-fluoro-deschloroketamine. These compounds show fragment mass of 222.1 *m/z*, present as a single peak

on the DART mass spectrum. There are several other compounds showing mass fragment of 222.1 *m/z*, such as 4'-Fluoro-α-pyrollidinopropiophenone, N-benzyl-N-phenylpropanamide, or N-(-phenylisopropyl)benzaldimine. In order to certainly identify the compound present in the sample analyzed, the GC-MS confirmatory method was applied. In this case, a standard of the suspected compound, such as the 2-fluorodeschloroketamine, was run alongside the unknown sample on the GC-MS to compare mass spectra and retention times.

For sample 20210226-001 "2", the highest peak showed a mass of 287.2 *m/z* (Figure 17). Performing a library search, the compound was identified as 2-methyl-AP-237, para-methyl-AP-237, or AP-238. It also indicated the potential for the 287.2 *m/z* peak to correspond to some cannabinoids, methyldienolone, or 4-hydroxytestosterone. In this case, a standard of the suspected compound, such as the AP-238, was run alongside the unknown sample on the GC-MS to compare mass spectra and retention times.

The screening results of the GC-MS further indicated that neither powder sample contained bucinnazine. The screening was qualitative only, and utilized a library search, using the SWGDRUG library. The GC-MS results showed the presence of 2-fluorodeschloroketamine and deschloroketamine in sample 20210226-001 "1" (Figure 18). The presence of the 2-fluorodeschloroketamine supports the results found in the DART-MS screen, and so suggests that this is likely the main compound present in sample 20210226-001 "1".

A standard of 2-fluorodeschloroketamine was obtained and run alongside the unknown powder in order to confirm the identity by comparing the retention times and the fragments of the mass spectra. Both compounds had a retention time of 8.3 minutes and had specific fragments of 193 *m/z* and 164 *m/z* (Figure 19, Figure 20).

The second unknown powder sample, 20210226-001 “2”, showed one peak on the GC-MS chromatogram, and was presumptively identified as AP-238, an analog of bucinnazine and a structural isomer of 2-methyl-AP-237 (Figure 21). This also correlates well with the peak observed in the DART-MS screen and suggests that one of these bucinnazine isomers is the main compound present in this sample.

A standard of AP-238 was obtained and run alongside the unknown powder in order to confirm the identity by comparing the retention times and the ions of the mass spectra of each. Both compounds had a retention time of 12.8 minutes and had specific fragments of 286 *m/z,* 186 *m/z,* and 117 *m/z* (Figure 22, Figure 23).

DISCUSSION

The unknown powders were presumptively identified as not being bucinnazine. The individual who submitted the powders to VCU Health indicated that the powders had been advertised as AP-237, a research chemical, but had been purchased from two different sources and caused two wildly different experiences upon consumption. This is a strong indicator of the dangers of the absence of proper labeling and quality control of substances commercialized on the black market. When the unknown powders were run alongside samples obtained of the potentially identified compounds, a retention time comparison was able to be performed on both the GC-MS and the mass spectra of the unknown powders and the obtained standards were compared. The identification of the compounds were possible after comparisons with the standard's retention time, and mass spectra. The samples were not able to be run on the LC-MS/MS method developed for bucinnazine, as no bucinnazine was identified in the powders. In the future, however, the method will be adjusted to include AP-238, and so the second powder can be run on the LC-MS/MS method.

The bucinnazine metabolites that were identified on the GC-MS and confirmed on the LC-MS/MS, 1-cinnamylpiperazine and 1-butyryl-4-(4-hydroxycinnamyl)piperazine, were consistent with some metabolites that had been mentioned previously in the literature. Additionally, it was shown that the amount of bucinnazine decreased over time, and the amount of 1-cinnamylpiperazine and 1-butyryl-4-(4-hydroxycinnamyl)piperazine increased over time, as their peak areas increased. Quantitation of 1-cinnamylpiperazine and 1-butyryl-4-(4-hydroxycinnamyl)piperazine will be performed as future work, as standards of each will need to be obtained in order to confirm and quantify them in the samples. The fact that no metabolites

were observed in the acid extracted samples run on the GC-MS could be for several reasons. The most likely reason is that the method used on the GC-MS was not sufficient for the acid metabolites. Improper volatilization or poor timing and temperatures could be the problem, and so further work on this method would need to be completed to see if the method can be adjusted. To do this, standards of 1-butyryl-4-(4-hydroxycinnamyl)piperazine, 1-cinnamylpiperazine, 1-(4-hydroxycinnamyl)piperazine, benzoic acid, 4-hydroxybenzoic acid, hippuric acid, and 4-hydroxyhippuric acid will be obtained and run on the GC-MS to optimize the method, and then the metabolized bucinnazine samples can be run alongside the standards to see if any of the acid metabolites are present. None of the acid metabolites were able to be observed on the LC-MS/MS for similar reasons as on the GC-MS, but additionally, the acid metabolites would need to be run on in negative mode on the LC-MS/MS, because acids donate protons, which means that they have a negative ion added to them within the ionization port of the instrument. This will be tested in future work on the metabolic pathway of bucinnazine, as well as the introduction of standards for all the expected metabolites for retention time comparison and eventual quantitation.

Quantitation of the bucinnazine in the metabolite samples needs to be reevaluated. Future work that will be completed on the metabolism of bucinnazine will therefore include the development of a cleaning procedure for the metabolized bucinnazine samples.

CONCLUSION

The overarching goal of this project was to develop a method for the identification of the new synthetic opioid, bucinnazine. Ultimately, this goal was successful, as an LC-MS/MS method that is rapid and effective can now be utilized on samples suspected to contain bucinnazine to provide a confirmatory identification and subsequent quantitation. This project demonstrated that substances advertised as bucinnazine are present in the black market, as shown with the obtained samples that were suspected to contain bucinnazine. However, these advertisements and samples are not necessarily accurate, as neither of the samples contained bucinnazine as was expected. This indicates just how much of a threat bucinnazine, and the opioid crisis are to public health due to the lack of proper labeling, and the risk of the presence of substances not described. It was also demonstrated that bucinnazine metabolites can potentially be utilized to identify bucinnazine.

Future work on the metabolic profile of bucinnazine will be performed by first optimizing the *in vitro* method utilized to form the metabolites. Further quantitation of the potential bucinnazine metabolites will be performed. Then studies on which specific enzyme metabolizes bucinnazine will be performed. Finally, *in vivo* studies on the metabolic profile of bucinnazine will be performed using animal models. The LC-MS/MS method that was validated in this study will be expanded to include the bucinnazine analogs, and then the unknown powder samples will be run on the LC-MS/MS method to both identify and quantitate the compounds in the powder samples.

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CRITICAL DATA

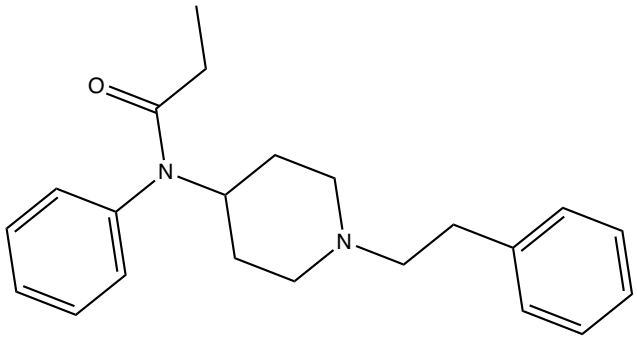

Figure 1. Chemical structure of Fentanyl.

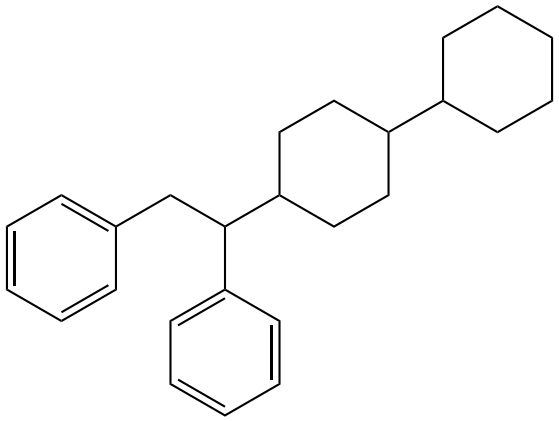

Figure 2. Chemical structure of MT-45.

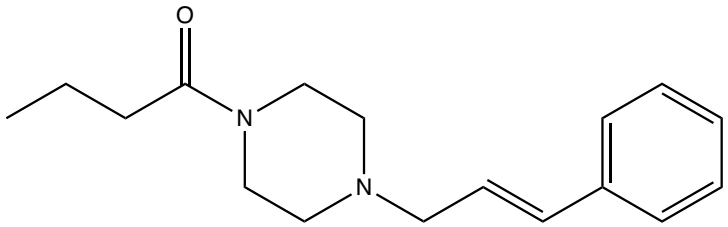

Figure 3. Chemical structure of bucinnazine (1-butyryl-4-cinnamylpiperazine).

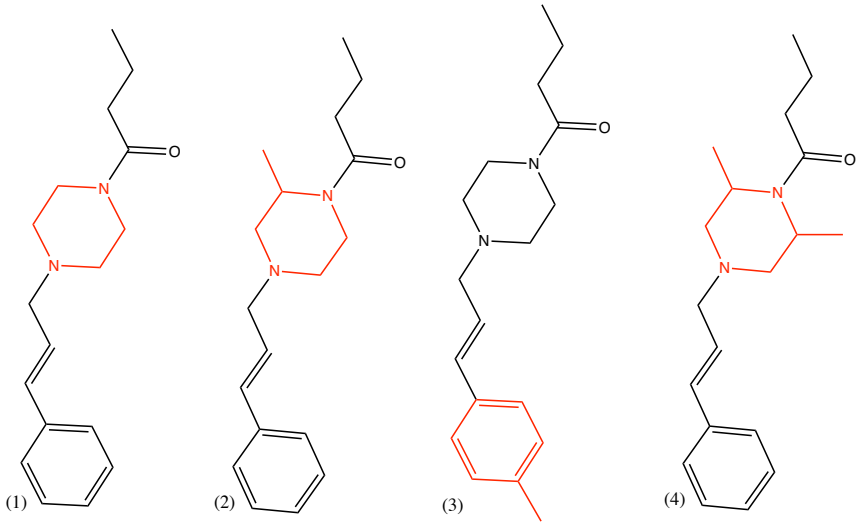

Figure 4. Chemical structures of bucinnazine (1), 2-methyl AP-237 (2), *para*-methyl AP-237 (3) and AP-238 (4).

The changes in the structures are shown in red.

Table 1. A table of the transition ions, collision energies, and pre-biases utilized on the LC-MS/MS.

Standard	Transitions (m/z)	Q1 Pre Bias (V)	Collision Energy (eV)	Q3 Pre Bias (V)
Bucinnazine	273 > 117	-30	-21.2	-10
	273 > 91	-14	-58.3	-30
	273 > 65	-14	-64.9	-26
Fentanyl-D5	342 > 105	-27	-40.5	-18
	342 > 188	-26	-25.2	-11

Table 2. Parameters analyzed for method validation

Parameter	Acceptance Criteria
Calibration Model	Linear, $r^2>0.999$
% Bias	$\pm$ 20% at each concentration
Within-Run Precision	%CV $\leq$ 20% at each concentration
Between-Run Precision	%CV $\leq$ 20% at each concentration
Carryover	Highest fortified concentration at which no carryover is observed
Limit of Detection	$(3.3*s_y)/Avg_m$
Lower Limit of Quantitation	$(10*s_y)/Avg_m$

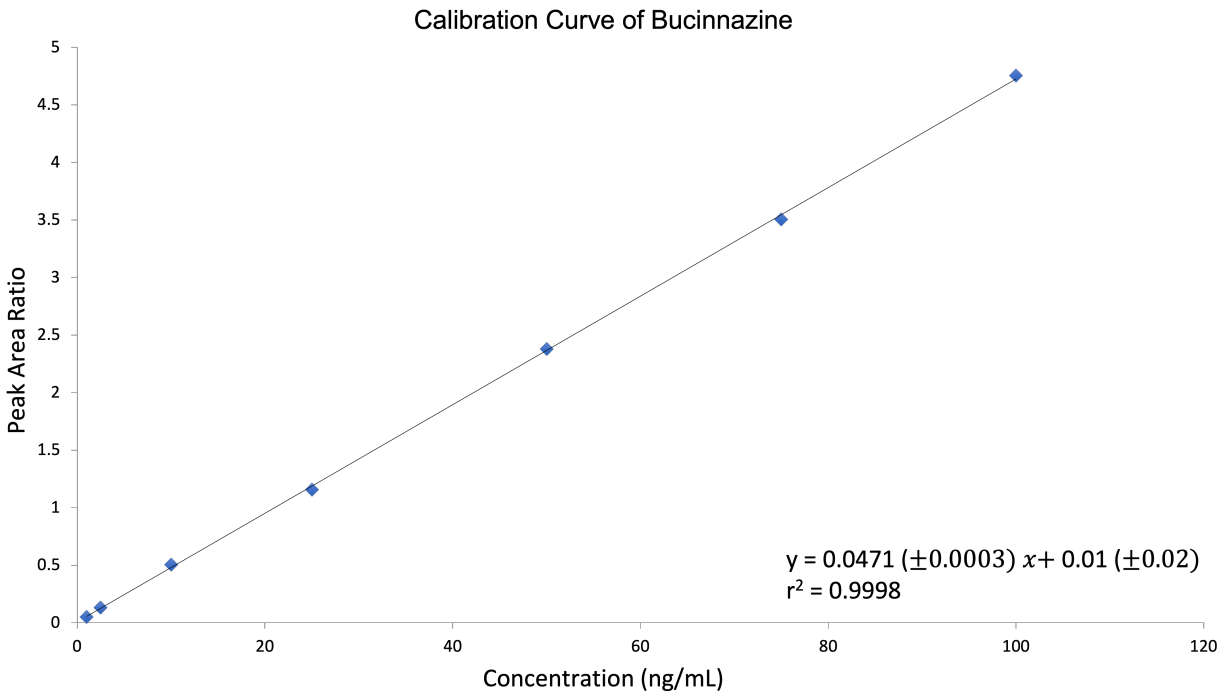

Figure 5. Calibration curve of bucinnazine run over three separate days and averaged.

Table 3. Table depicting the standardized residuals used to evaluate the calibration model.

Standard (n=3)	Concentration (ng/mL)	Observed Peak Area Ratio	Predicted Peak Area Ratio	Standardized Residuals
1	1	0.05	0.05	-0.06
2	2.5	0.13	0.13	0.05
3	10	0.50	0.45	0.05
4	25	1.16	1.19	-0.03
5	50	2.38	2.37	0.005
6	75	3.50	3.55	-0.01
7	100	4.75	4.73	0.006

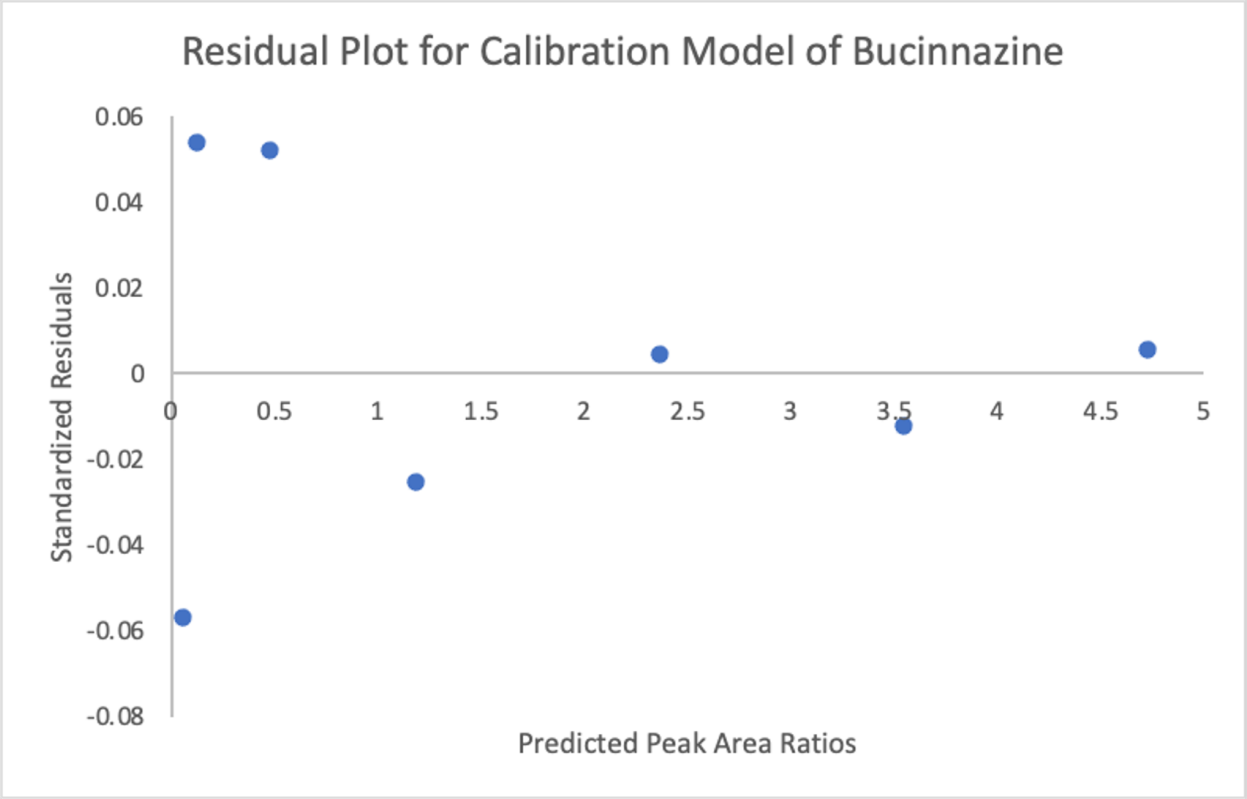

Figure 6. Residual plot to analyze the calibration model of bucinnazine.

Table 4. Table depicting the actual average concentrations of bucinnazine over the three-day validation runs for the calibration curve, with the associated bias.

Standard (n=3)	Theoretical Concentration (ng/mL)	Actual Concentration (ng/mL)	% Bias
1	1	0.94	-6.38
2	2.5	2.65	5.80
3	10	10.53	5.35
4	25	24.38	-2.49
5	50	50.25	0.50
6	75	74.13	-1.16
7	100	100.63	0.63

Table5. Table depicting the average concentration, standard deviation, and %CV obtained for the within-run analysis of QC samples over three different days.

Within Run Precision Day 1

QC Sample	Theoretical Concentration (ng/mL)	Average Actual Concentration (ng/mL)	Standard Deviation	%CV
Low (n=3)	3	2.46	0.06	2.59
Mid (n=3)	30	24.74	0.35	1.40
High (n=3)	80	68.53	0.38	0.56

Within Run Precision Day 2

QC Sample	Theoretical Concentration (ng/mL)	Average Actual Concentration (ng/mL)	Standard Deviation	%CV
Low (n=3)	3	3.14	0.04	1.21
Mid (n=3)	30	31.98	1.01	3.14
High (n=3)	80	83.33	0.35	0.42

Within Run Precision Day 3

QC Sample	Theoretical Concentration (ng/mL)	Average Actual Concentration (ng/mL)	Standard Deviation	%CV
Low (n=3)	3	3.03	0.27	8.93
Mid (n=3)	30	34.34	0.76	2.21
High (n=3)	80	89.23	0.63	0.71

Table 6. Table depicting the average concentration, standard deviation, and %CV obtained for the between-run analysis of QC samples over three different days.

Between Run Precision

QC Sample	Theoretical Concentration (ng/mL)	Average Actual Concentration (ng/mL)	Standard Deviation	%CV
Low (n=9)	3	2.87	0.30	10.36
Mid (n=9)	30	30.35	4.09	13.46
High (n=9)	80	80.36	8.71	10.84

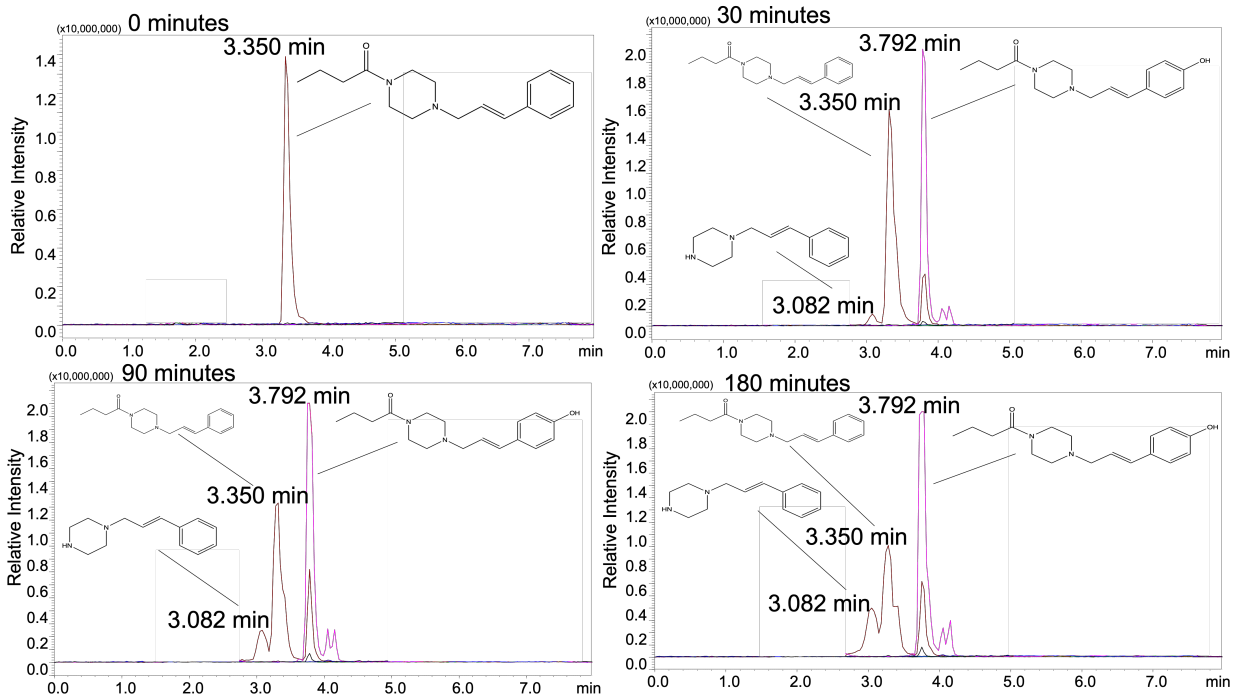

Figure 7. Time course study of the formation of bucinnazine metabolites *in vitro*.

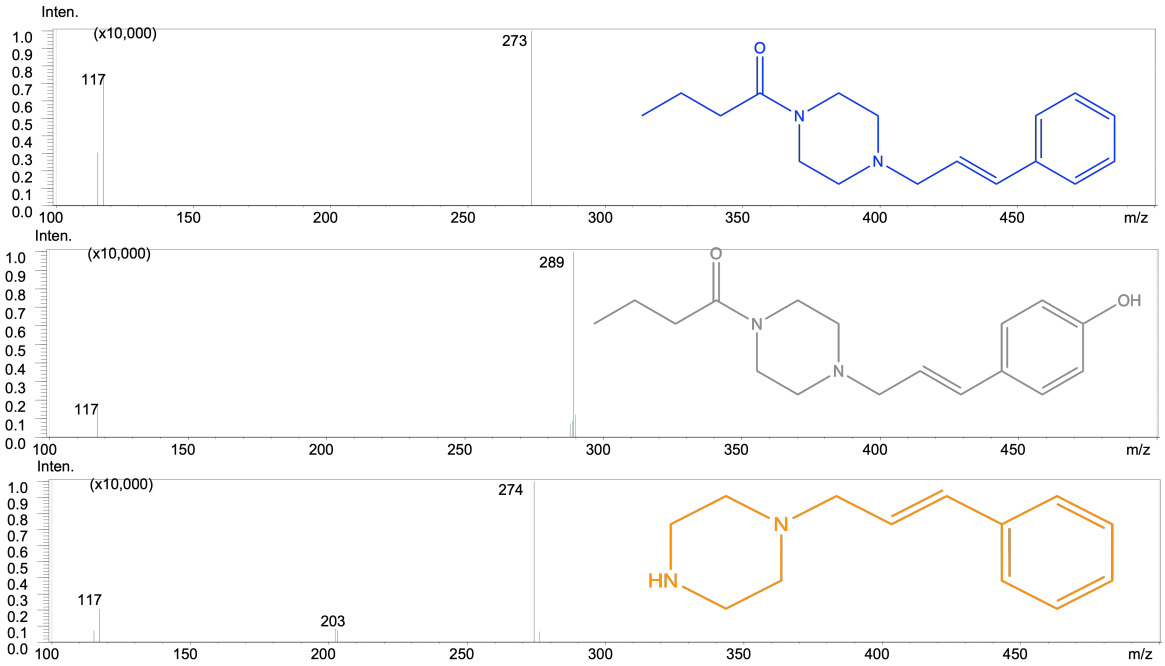

Figure 8. LC-MS/MS mass spectra of bucinnazine (top), 1-butyryl-4-(hydroxycinnamyl)piperazine (middle), and 1-cinnamylpiperazine present in a metabolized sample.

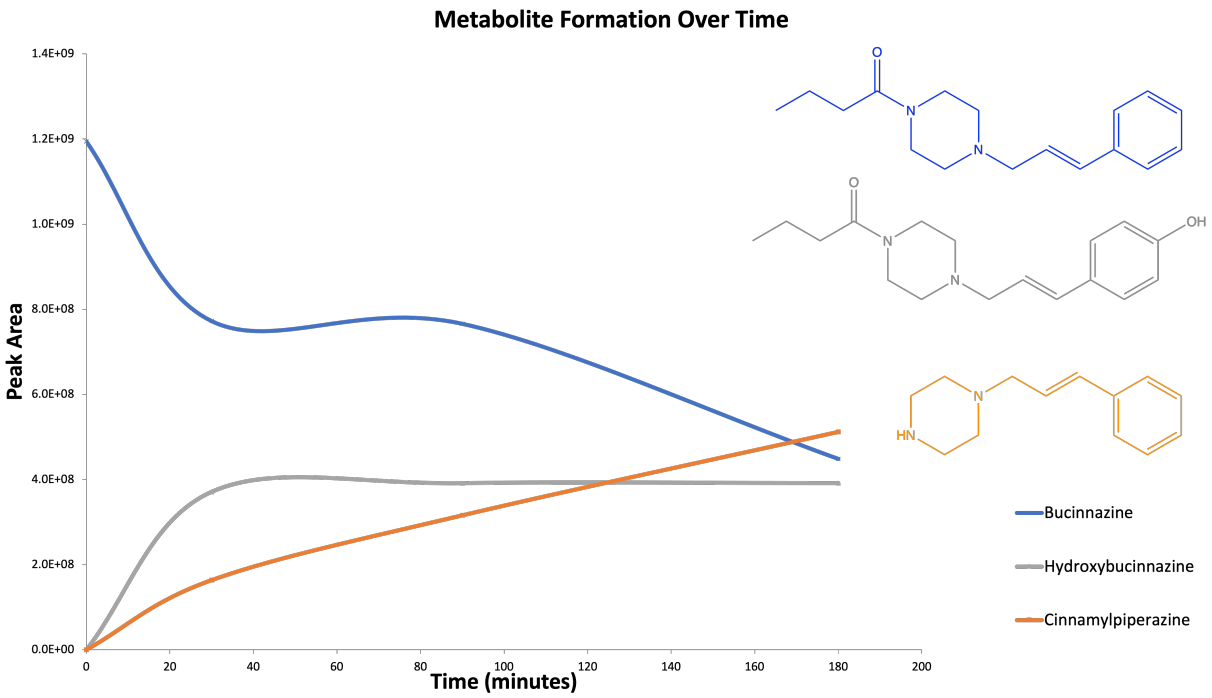

Figure 9. Graph showing the formation of bucinnazine metabolites *in vitro* over time.

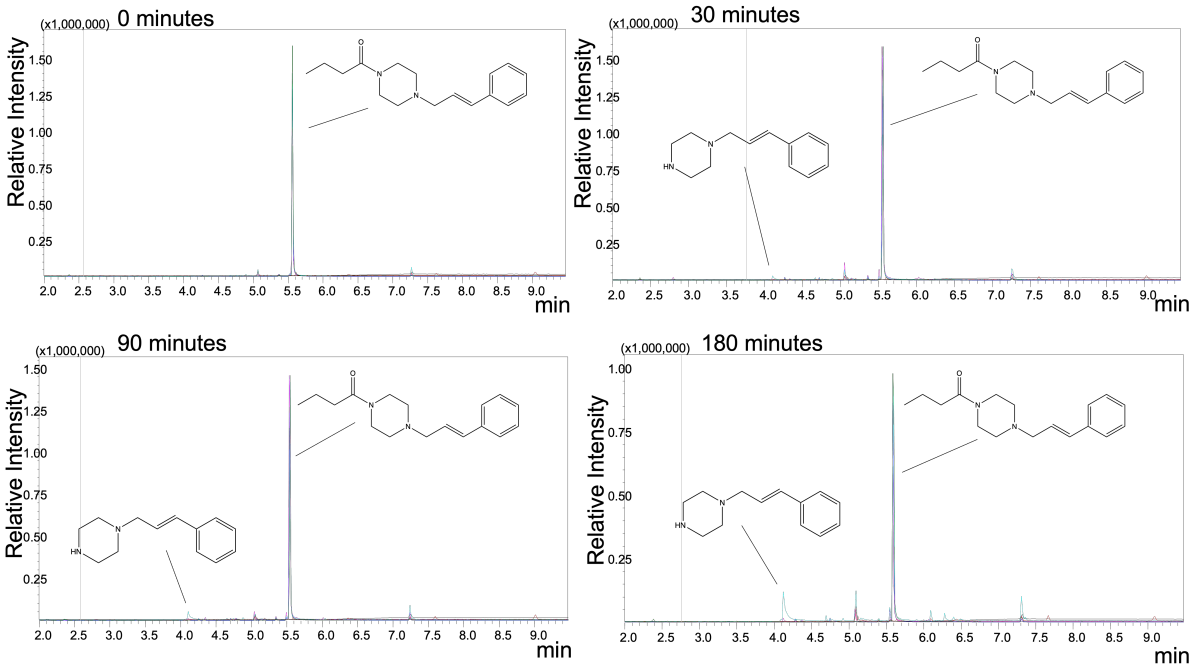

Figure 10. Time course study of the formation of bucinnazine metabolites *in vitro*. Ions listed in the top left are the selected ions corresponding to bucinnazine and its expected metabolites.

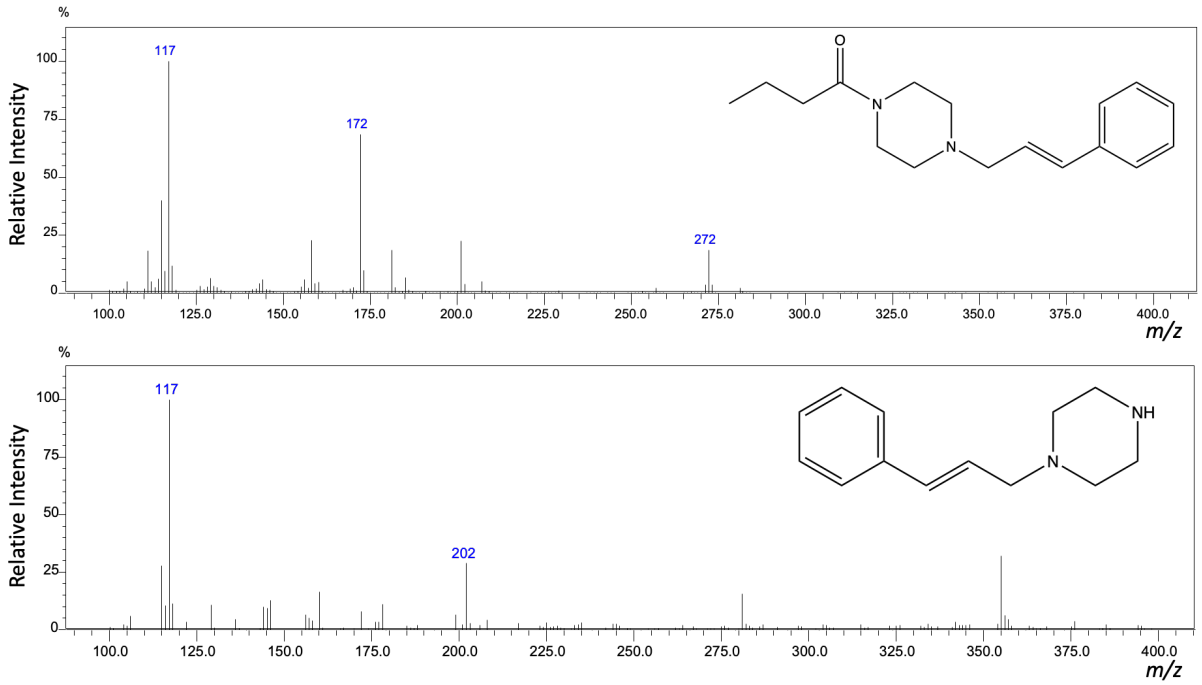

Figure 11. Mass spectra of bucinnazine (top) and 1-cinnamylpiperazine (bottom) obtained from the GC-MS run of the bucinnazine metabolite samples.

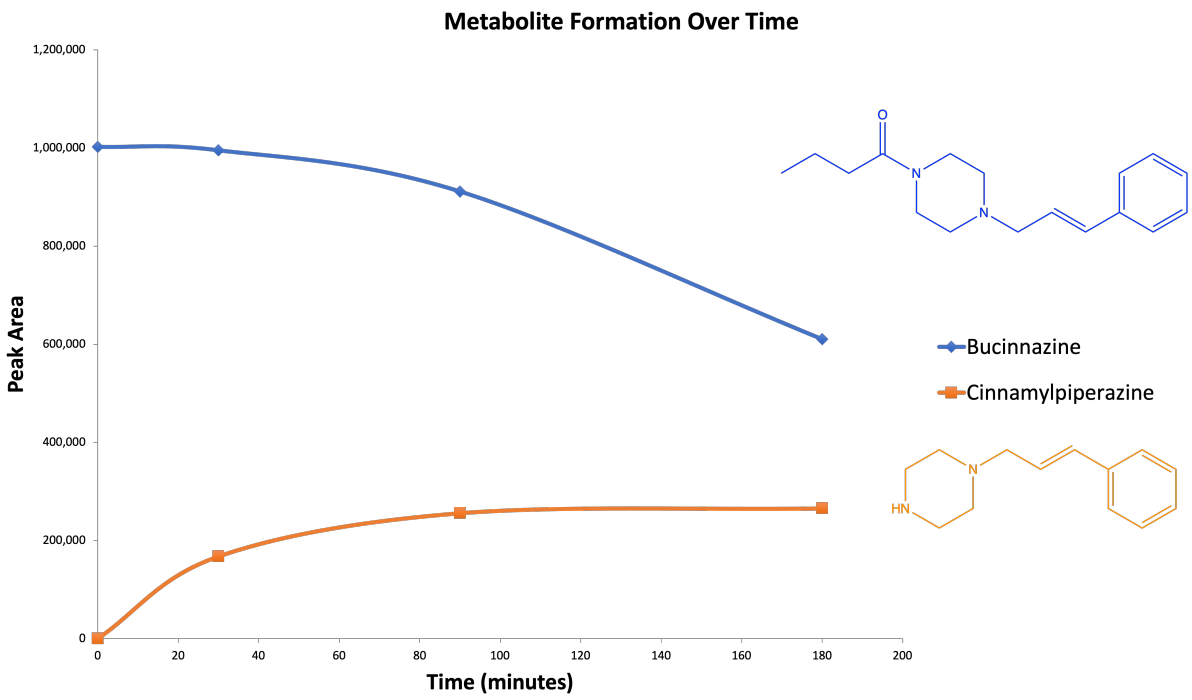

Figure 12. Graph depicting the formation of bucinnazine metabolites *in vitro* over time.

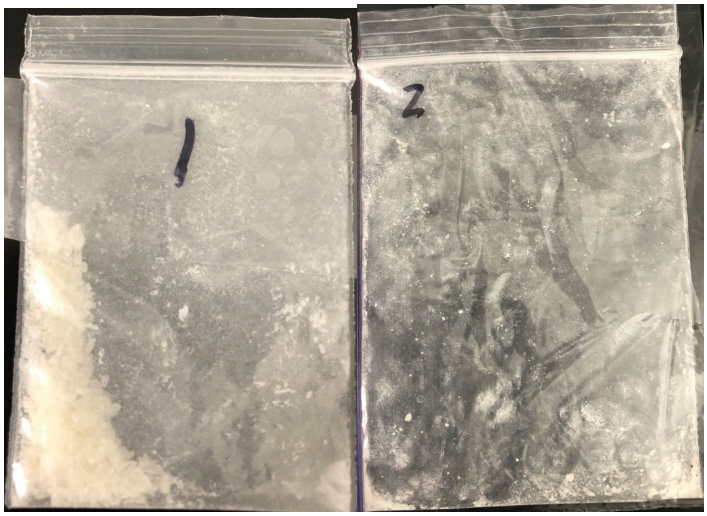

Figure 13. Images of the two obtained unknown powder samples suspected to be bucinnazine. Right: 20210226-001 "1"; Left: 20210226-001 "2".

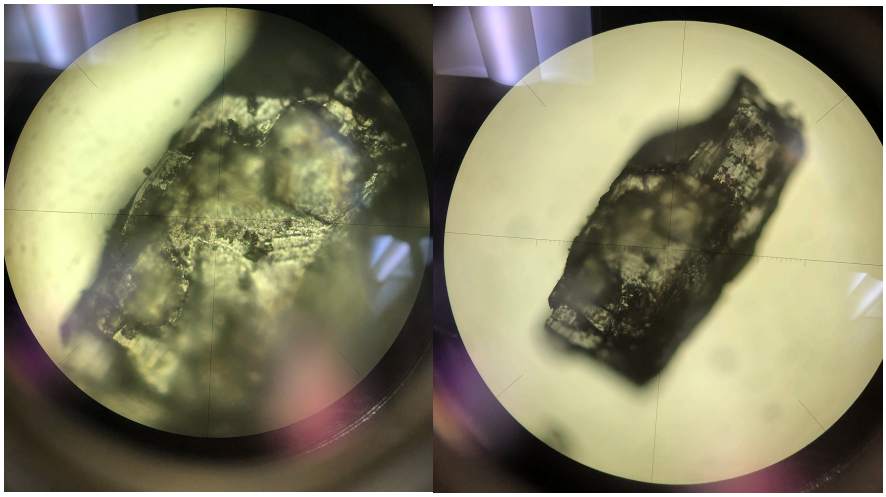

Figure 14. Images of 20210226-001 “1” under the microscope. Right: 100x; Left: 200x.

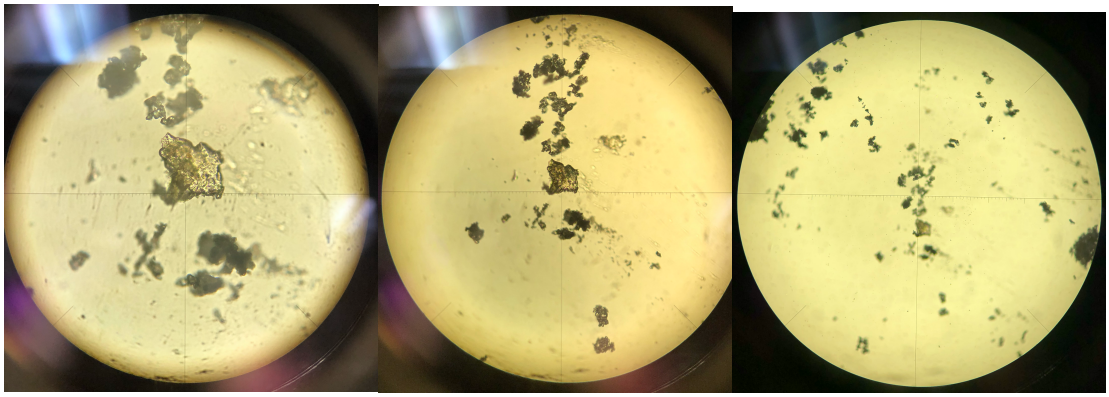

Figure 15. Images of 20210226-001 “2” under the microscope. Right: 100x; Middle: 200x; Left: 400x.

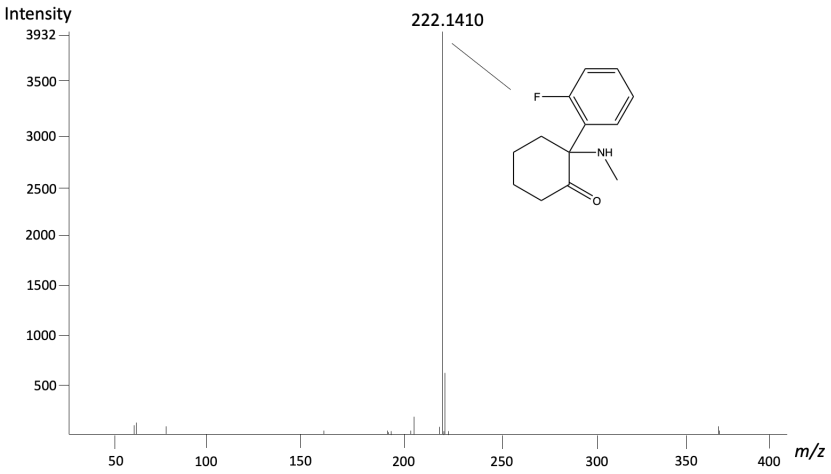

Figure 16. DART-MS screening results for unknown powder 20210226-001 “1”. The chemical structure depicted on the figure is the suspected compound, 2-fluorodeschloroketamine.

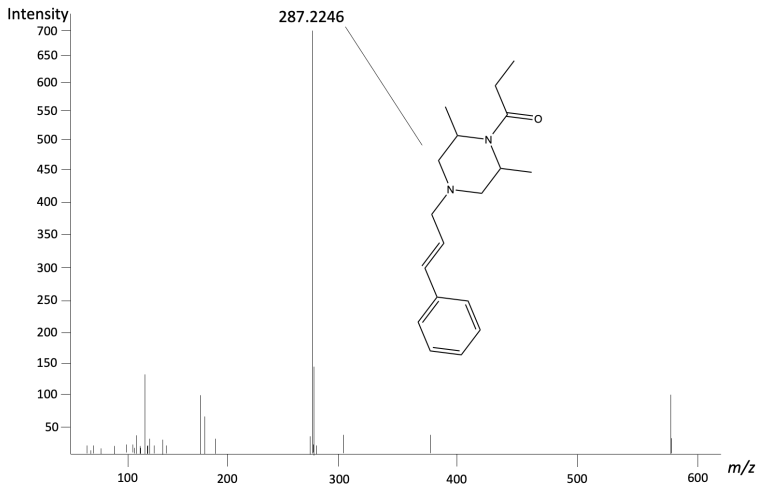

Figure 17. DART-MS screening results for unknown powder 20210226-001 “2”. The chemical structure depicted on the figure is the suspected compound, AP-238.

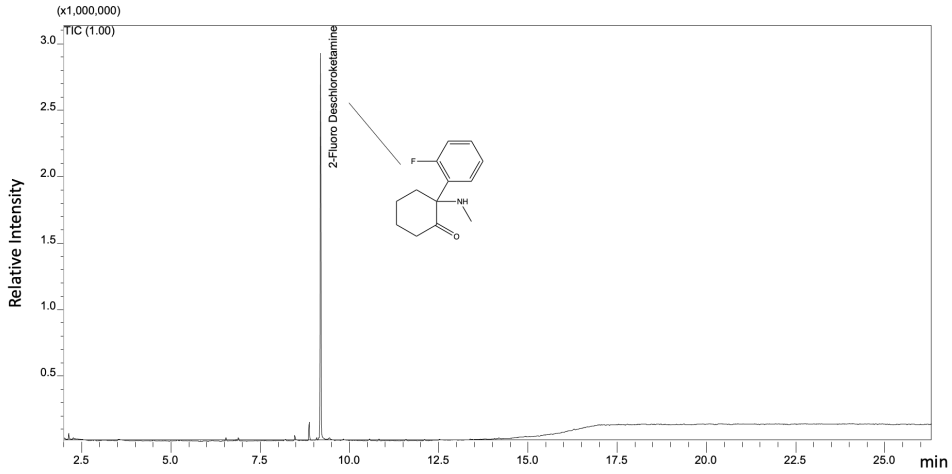

Figure 18. GC-MS screening results for unknown powder 20210226-001 "1". The chemical structure depicted on the figure is 2-fluorodeschloroketamine, which corresponds to the library results of the GC-MS screening.

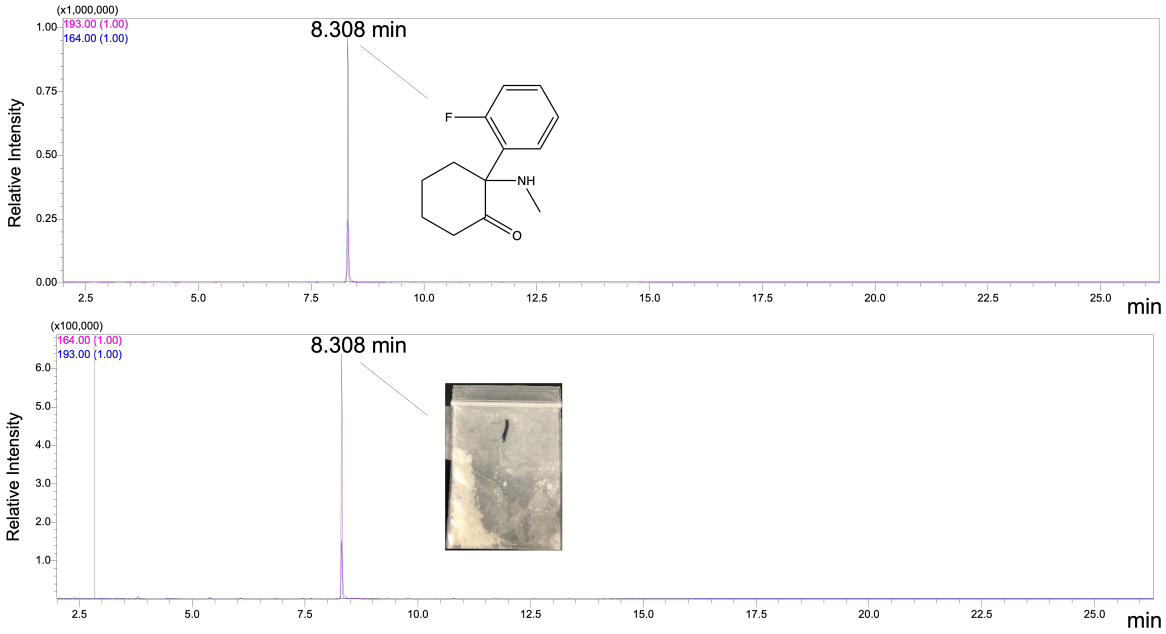

Figure 19. GC-MS screening results for unknown powder 20210226-001 "1". The top chromatogram is that of the 2-fluorodeschloroketamine standard, and the bottom chromatogram is that of 20210226-001 "1".

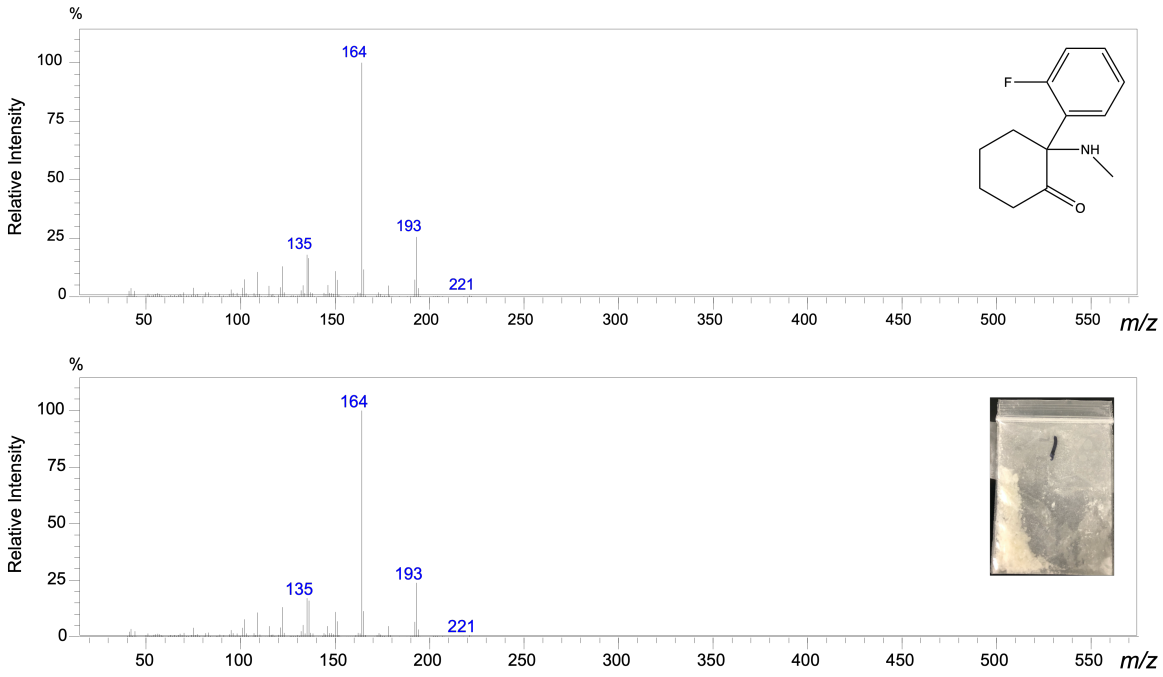

Figure 20. GC-MS screening results for unknown powder 20210226-001 "1". The top mass spectrum is that of the 2-fluorodeschloroketamine standard, and the bottom mass spectrum is that of 20210226-001 "1".

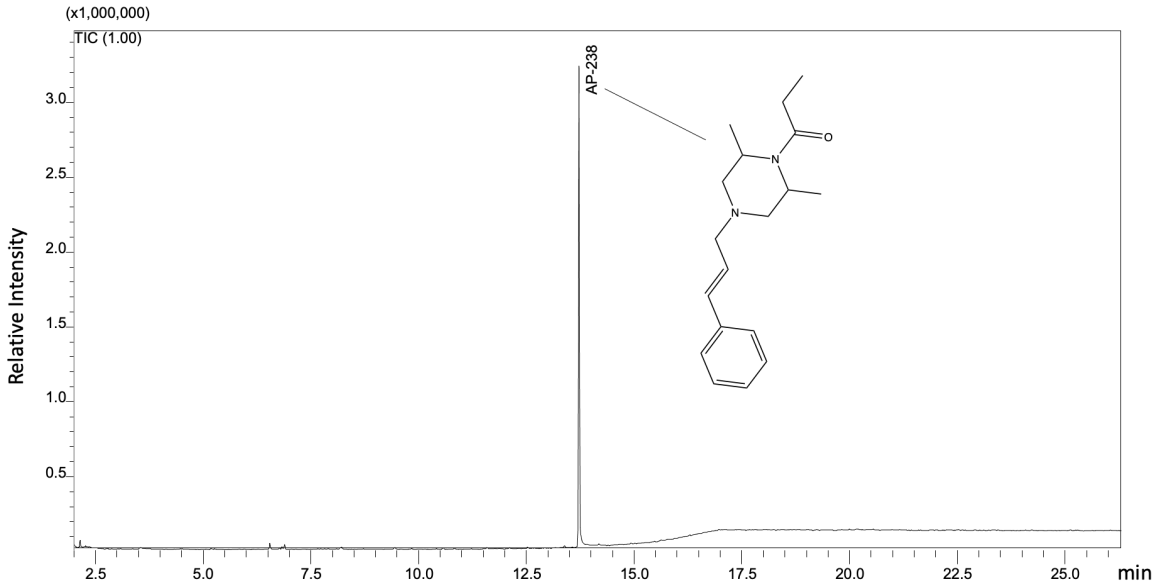

Figure 21. GC-MS screening results for unknown powder 20210226-001 "2". The chemical structure depicted on the figure is AP-238, which corresponds to the library results of the GC-MS screening.

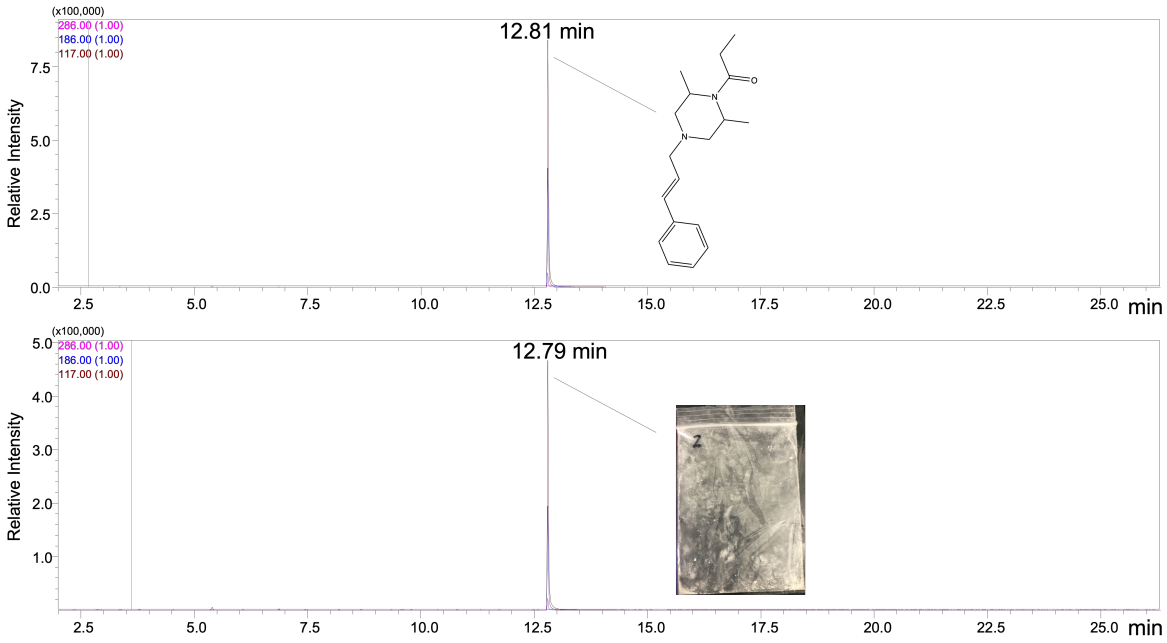

Figure 22. GC-MS screening results for unknown powder 20210226-001 "2". The top chromatogram is that of the AP-238 standard, and the bottom chromatogram is that of 20210226-001 "2".

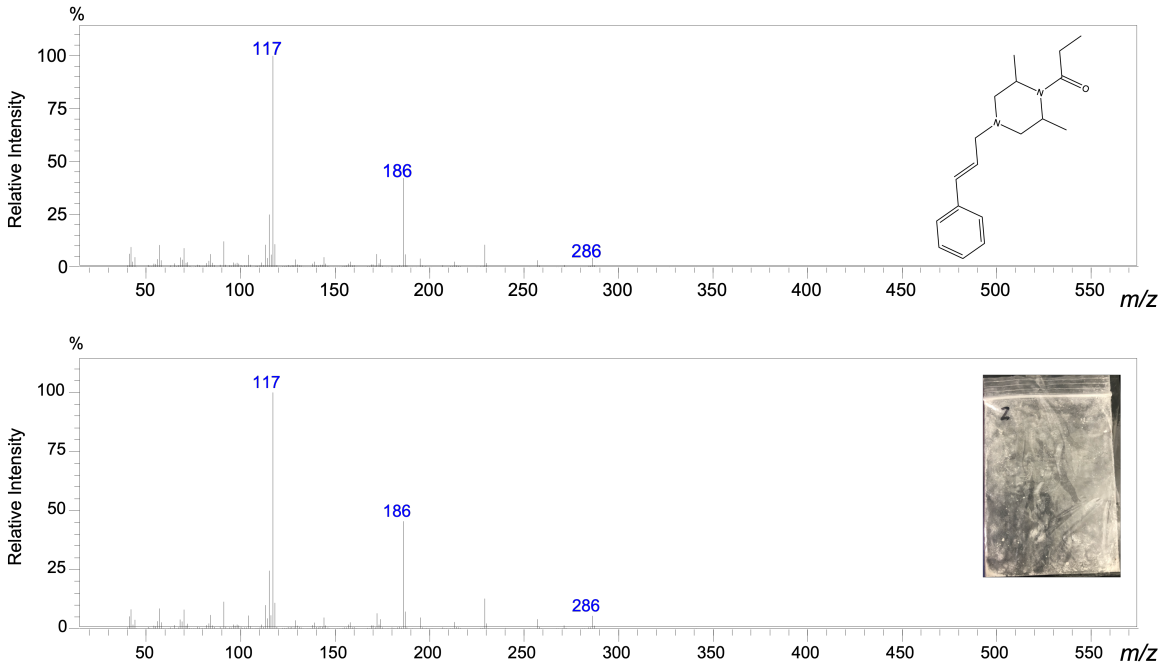

Figure 23. GC-MS screening results for unknown powder 20210226-001 "2". The top mass spectrum is that of the AP-238 standard, and the bottom mass spectrum is that of 20210226-001 "2".

VITA

EDUCATION

Virginia Commonwealth University (VCU), Richmond, VA May 2022
Master of Science in Forensic Science, Concentration: Chemistry – Drugs and Toxicology, FEPAC accredited

Thesis: *Identification of the New Synthetic Opioid Bucinnazine using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)*
Thesis Advisor: Emanuele Alves, Ph.D.

University of Wyoming (UW), Laramie, WY December 2019
Bachelor of Science in Chemistry

RELEVANT SKILLS

- *Computer skills:* Microsoft Office Suite, Adobe PDF
- *Instrumentation:* LC-MS systems, GC-MS systems, UV-Vis, FTIR, and DART
- *Microscopy:* PLM, dark field, bright field, phase contrast, and comparison microscopy
- *General lab techniques:* buffer preparation, reagent preparation, proper handling and documentation of hazardous and controlled substances

EXPERIENCE

Research Assistant, VCU (Department of Forensic Science) January 2021-Present

- Helped establish a new research laboratory and assisted with QA/QC and safety practices
- Trained new research assistants on standard operating procedures and general lab protocol
- Performed routine maintenance and repairs on GC-MS, LC-MS, and Chemical Analyzer

Graduate Teaching Assistant, VCU (Department of Forensic Science) August 2020-Present

- Courses taught include Microscopy, Paints and Polymers, Forensic Chemistry, Forensic Toxicology, Instrumentation, and Fire Debris Analysis
- Designed laboratory protocols to improve class learning outcomes and increase hands-on experience
- Responsible for chemical fume hood maintenance and teaching instrument and equipment maintenance, including GC-MS, LC-MS, FTIR, UV-vis, Chemical Analyzer, centrifuges, balances, and pipettes

Research Assistant, UW (Department of Chemistry) August 2018-December 2019

- Extracted lipids from bacteria and used a MALDI-TOF-MS instrument for research and data analysis

Intern, Colorado Bureau of Investigation (CBI) May 2018-August 2018

- Gathered and statistically analyzed serological data to determine the efficiency of forensic laboratory testing methods, and utilized presumptive tests to determine workflows to lessen consumption of evidence
- Validated a urobilinogen test prior to casework implementation

PUBLICATIONS AND PRESENTATIONS

Resnik, K. N.; Alves, E. A. February 2022. *In Vitro* Metabolic Profile of a New Synthetic Opioid Bucinnazine Using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). 74th Annual AAFS Scientific Conference. Seattle, WA.

Resnik, K.; Brandão, P.; Alves, E. A. DARK Classics in Chemical Neuroscience: Bucinnazine. *ACS Chem. Neurosci.* 2021. https://doi.org/10.1021/acschemneuro.1c00522.

Rogers CE, **Resnik KN**, Troudt RD, Trahey SA, Miller SL. August 2018. Improving the efficiency of sexual assault kit processing by evaluating the predictive value of acid phosphatase and prostate specific antigen testing. 103rd IAI International Educational Conference. San Antonio, TX.

GRANTS AND FELLOWSHIPS

Idea Network for Biomedical Research Excellence (INBRE) Research Fellowship (UW) 2019

PROFESSIONAL MEMBERSHIPS

Phi Beta Kappa Honor Society	March 2018
American Academy of Forensic Science (AAFS), Student Affiliate	February 2022
Society of Forensic Toxicologists (SOFT), Student Member	March 2022